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STUDIES OF ADRENAL NUCLEIC ACIDS: THE INFLUENCE OF ACTH, UNILATERAL ADRENALECTOMY AND GROWTH HORMONE UPON ADRENAL RNA AND DNA IN THE DOG[1]

EDWIN D. BRANSOME, JR. AND WILLIAM J. REDDY

Departments of Medicine, Harvard Medical School and Peter Bent Brigham Hospital, Boston, Massachusetts

ABSTRACT

Ribonucleic acid (RNA) was shown to be increased in the canine adrenal cortex after *in vivo* ACTH administration; the greatest RNA increase was in the microsomal fraction. The gain in size and weight of the adrenal gland after ACTH was not accompanied by a significant change in desoxyribonucleic acid (DNA), indicating that cell hypertrophy rather than cell proliferation had taken place. After growth hormone administration or unilateral adrenalectomy, adrenal gland DNA increased; increase in adrenal RNA was much less than after ACTH. Growth hormone and unilateral adrenalectomy, then, had a corticotropic influence qualitatively dissimilar to ACTH.

THE influence of the anterior pituitary on the adrenal cortex was established by Smith thirty years ago (1). The principal factor has proved to be a polypeptide hormone, ACTH (2) which stimulates glucocorticoid biosynthesis and secretion (3, 4, 5). The increase in adrenal cortical weight (AW) (6, 7, 8), and the depletion of adrenal ascorbic acid (AA) (9): have both been employed in assay of ACTH potency. Differences in the AW and AA potency of various ACTH preparations (10, 11, 12) and between AA and steroidogenic potency (13) have reinforced other speculations on the existence of more than one adrenocorticotropin (14–17).

Other factors affecting adrenal morphology provide further examples of the dichotomy of corticotrophic influences. The steroid secretion may be decreased in rat adrenals that are hypertrophied after high diencephalic lesions (18). Anterior pituitary growth hormone (2) increases adrenocortical weight (19, 20) and mitotic activity (8) in the hypophysectomized

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animal; it and ACTH synergistically affect the zona fasciculata (21). Growth hormone also increases the cortex weight in the intact animal (22, 23), apparently not affecting glucocorticoid secretion (24, 25), but stimulating aldosterone secretion by the zona glomerulosa (26). The increase in adrenal weight fostered by pregnancy plasma (27) is probably due to high growth hormone levels (28, 29). Thyroid hormones exert a hypertrophic influence on the zona fasciculata that appears to be mediated through the pituitary, perhaps by ACTH (30, 31). Androgenic steroids have been found to be capable of partially maintaining the adrenal cortex after hypophysectomy (32, 33) but suppress steroid secretion (34, 35). Estrogenic steroids exert a corticotrophic influence in the presence of the pituitary (36–38) but do not themselves stimulate steroid secretion (39–41). After unilateral adrenalectomy there is compensatory hypertrophy of the remaining adrenal that depends upon an intact pituitary (42, 43). Mitotic activity in the cortex is increased by this stimulation (44); the adrenal medulla is unaffected. Glucocorticoids (45), androgens (46), hypothyroidism (47), and lesions of the median eminence of the hypothalamus (48) may inhibit compensatory hypertrophy, as estrogen stimulation may potentiate it (46, 49). Such data, and the report of increased plasma corticoids after adrenal enucleation (50) implicate ACTH as the important trophic factor, but there is indirect evidence that there may be other factors (51) and that ACTH indeed may not be the major factor involved (52).

The purpose of this study is to investigate the effects of ACTH, of growth hormone, and of unilateral adrenalectomy on net synthesis of nucleic acids. The amount of ribonucleic acid (RNA) in cells has been related to their metabolic activity: increased RNA has been correlated with endocrine activity of the thyroid (53), anterior pituitary (54), and the adrenal cortex. Increased desoxyribonucleic acid (DNA) indicates the occurrence of cell proliferation. Changes in adrenal nucleic acids would thus provide a biochemical basis for comparison of the effects of these stimuli on the adrenal cortex.

MATERIALS AND METHODS

Normal male dogs were boarded in identical cages in the same room at the Harvard Medical School animal farm. They were fed a standard diet of Purina fox cubes and horse meat, with water *ad libitum*. Dogs were selected at random to be in one of four groups:

A. Normal controls (9 dogs).
B. ACTH (5 dogs) treated: 40 units Upjohn Depo-ACTH (lot 4734) was injected intramuscularly twice daily for seven days.
C. Growth hormone (4 dogs) treated: 2 mg. Armour bovine growth hormone (lot G61406) was injected intramuscularly twice a day for one week.
D. Unilateral adrenalectomy (4 dogs); one week after surgical removal of the left adrenal gland, the right adrenal was removed and compared with the first.

Total Adrenal Nucleic Acids

After one week of treatment or observation, the dogs were sacrificed with an overdose of intravenous nembutal. On most dogs a 24-hour urine collection for 17-ketosteroids (55, 56) and 17-hydroxycorticosteroids (57) was made the day before termination of the experiment. The adrenal glands were quickly removed and frozen. Between 18 and 48 hours later, 10 ml. of distilled water were added per gram of adrenal, and the adrenal glands were homogenized in a Waring blendor or Potter Elvehjem homogenizer. The RNA and DNA contents were determined by the method of Schneider (58) which is based on differential measurement of pentose and desoxypentose with orcinol and diphenylamine reactions (59). Yeast RNA and calf thymus DNA were used as standards. All measurements were made in triplicate. DNA and RNA standards (obtained from the Nutritional

TABLE 1. RELATIVE OPTICAL DESNITY OF STANDARD COMPOUNDS IN THE DIPHENYLAMINE REACTION

DNA			D-2-desoxyribose			D-ribose		
μg/ml.	OD*×10^3	OD×10^3/μg	μg/ml.	OD×10^3	OD×10^3/μg	μg/ml.	OD×10^3	OD×10^3/μg
20	27	1.4	5	38	7.6	5	9.5	1.9
40	64	1.6	10	82.5	8.3	10	10.0	1.0
60	87	1.5	20	169	8.5	20	7.0	0.4
100	140	1.4	40	327.5	8.2	40	13.5	0.2
Average		1.5			8.2			

* OD: Optical Density Units.

TABLE 2. RELATIVE OPTICAL DENSITY OF STANDARD COMPOUNDS IN THE ORCINOL REACTION

RNA			DNA			D-ribose			D-2-desoxyribose		
μg/ml.	OD*×10^3	OD×10^3/μg	μg/ml.	OD×10^3	OD×10^3/μg	μg/ml.	OD×10^3	OD×10^3/μg	μg/ml.	OD×10^3	OD×10^3/μg
10	90	9.0	10	15	1.5	5	215	43.0	5	31	6.2
20	175	8.8	20	30	1.5	10	445	44.5	10	57	5.7
40	345	8.6	40	55	1.4	20	910	44.5	20	87	4.4
60	520	8.7	60	80	1.3	40	1750	43.8	40	160	4.0
Average		8.8			1.4			44.2			5.1

* OD: Optical Density Units.

Biochemical Co., Cleveland, Ohio) were calibrated against desoxyribose and ribose standards. (Tables 1, 2) with 5% trichloracetic acid blanks on a Coleman Jr. spectrophotometer or Beckman B spectrophotometer. The "t-test" of significance of the difference between independent means (60), with 12 degrees of freedom, was applied to the data of ACTH and growth hormone studies. The "t-test" of significance of the difference between dependent means with 3 degrees of freedom was applied to the data of the unilateral adrenalectomy study.

Cortical Cell Fraction Nucleic Acids

Two male dogs were treated with ACTH as above. Two normal untreated dogs were sacrificed at the same time. The adrenal cortices were carefully dissected free from medulla and homogenized with a Potter-Elvehjem homogenizer in 9 ml. ice cold 0.25 molar sucrose per gram wet tissue. The homogenate was transferred to lusteroid tubes and was separated into subcellular fractions with refrigerated high speed centrifuge according to the method of Hogeboom and Schneider (61).

All fractions were washed with equal portions of chloroform:methanol. Nucleic acids were extracted with 5% trichloroacetic acid at 90° C for 15 minutes, and the RNA content of the eluate assessed by the orcinol reaction with correction of the "debris and nuclear fraction" for DNA content as indicated by the diphenylamine reaction.

RESULTS

The Effect of ACTH Administration

Administration of bovine ACTH to five normal male dogs (Table 3) resulted in gross increase of adrenal cortical width without discernible change in the amount of medullary tissue. The mean adrenal weight of the treated group was almost twice that of the untreated controls ($P < 0.001$). Evidence of the stimulatory effect of ACTH treatment was provided by increase in 24-hour urinary 17-ketosteroids (17-KS) and 17-hydroxycorticosteroids (17-OHCS) the day before sacrifice, and by increase in gross weight of the liver (62). There was no significant difference in the mean DNA content of the ACTH stimulated glands, 1.9 mg., from that of untreated controls, 1.7 mg.

TABLE 3

	No. dog	Body weight in kg.	Weight (gm.) one adrenal	DNA mg./ adr.	RNA mg./ adr.	24 hr. urine	
						17-KS	17-OHCS
Normal controls	1A	23.2	0.62	1.9	0.6	1.7	1.1
	2A	18.8	0.59	1.9	0.5	1.2	2.0
	8A	15.1	0.84	2.7	0.9	5.2	1.8
	4B	15.4	0.58	1.9	0.9	0.5	2.0
	6B	13.6	0.65	1.9	0.9	0.6	0.8
	U1	16.4	0.77	1.6	0.9		
	U2	25.4	0.67	1.6	0.6		
	U3	26.1	0.85	1.1	0.5		
	U4	16.3	0.53	1.1	0.8		
		Average	0.67	1.7	0.7	1.8	1.5
ACTH treated	3A	18.1	1.58	2.1	2.9	7.9	7.5
	4A	19.9	1.16	1.7	2.8	6.1	8.6
	7A	16.8	1.55	2.7	5.1	7.8	7.6
	1B	14.6	0.70	1.6	1.5	1.9	4.0
	2B	14.0	1.14	1.6	2.3	2.9	3.2
		Average	1.23	1.9	2.9	5.3	6.0
Growth hormone treated	G1	18.3	0.92	3.1	1.9	2.8	0.7
	G2	18.1	0.91	3.5	1.6	5.8	3.1
	G3	17.4	0.87	2.0	1.6	5.7	2.8
	G4	18.3	0.92	3.3	1.7	4.8	3.1
		Average	0.91	3.0	1.7	4.8	2.2
Unilateral adrenalectomy	U1	16.4	1.07	3.5	2.8	3.3	6.5
	U2	25.4	0.97	2.5	1.2	12.1	9.1
	U3	26.1	0.96	2.7	0.6	2.6	7.6
	U4	16.3	0.52	3.0	0.8	2.1	1.3
		Average	0.88	2.9	1.3	5.0	6.1

RNA content of the adrenals was increased more than four times by ACTH with a significant difference ($P < 0.001$) between the mean 2.9 mg. per adrenal gland and the mean 0.7 mg. of the control dogs. "Mitochondrial" RNA of the demedullated adrenals from two ACTH treated dogs was increased to about twice that of two untreated dogs. The greatest quantitative increase in RNA, however, was in the $80,000 \times g$ "microsomal" fraction (Fig. 1). In the untreated animals "microsomal" RNA accounted for 30% of total RNA, but after ACTH treatment comprised almost 50% of a doubled total RNA.

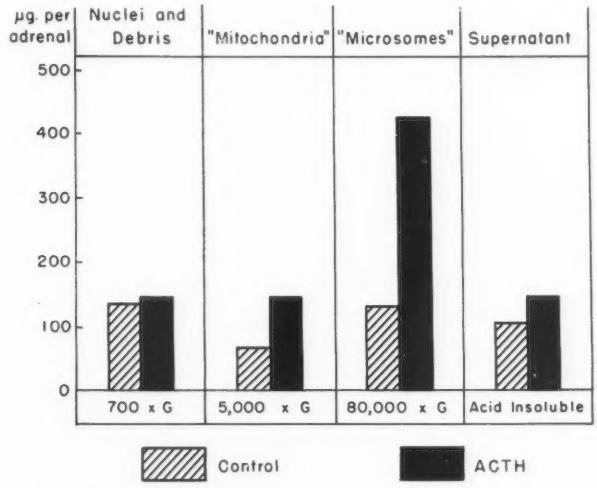

FIG. 1. RNA content in cell fractions of the adrenal cortex.

The Effect of Unilateral Adrenalectomy

Right adrenal glands removed from four dogs seven days after left adrenalectomy had less cortical thickening than in the ACTH-treated group; again, there were no apparent changes in medullary tissue. There were increases in the adrenal weight of the right glands of three dogs when compared to the previously removed left adrenals, but not in the fourth dog. Untreated control animals had no significant weight preponderance of either the left or right adrenals (Table 4), there were differences, however, of up to 15% between the weights of two adrenals from the same animal.

DNA content of the remaining right adrenal was about twice that of the previously removed left adrenal (Table 3). There was a significant average increase of 1.6 mg. ($P < 0.01$). Adrenal RNA was increased in two of the four dogs; a tendency that was not significant for the group, ($P > 0.21$).

The Effect of Growth Hormone Administration

The cortices of adrenal glands from four dogs treated with bovine growth

TABLE 4. CONTROL ADRENALS: LEFT VS. RIGHT

Dog	Left			Right		
	Weight	DNA	RNA	Weight	DNA	RNA
		mg./gland			mg./gland	
1A	0.65	2.0	0.7	0.59	1.8	0.5
2A	0.52	2.4	0.5	0.66	3.0	0.5
3A	0.88	1.9	0.8	0.80	1.9	1.0
4B	0.55	2.0	0.9	0.60	1.8	0.9
6B	0.62	1.7	1.0	0.68	2.1	0.8

Weight $P > 0.5$
$P > 0.5$
$P > 0.6$

hormone for seven days were thicker than normal; medullary size was unchanged. Mean adrenal weight in the growth hormone treated animals (Table 3) was 34% greater than in the control group; a significant increase ($P < 0.001$). Urinary 17-ketosteroid excretion was elevated in the growth hormone group; 17-hydroxycorticosteroid excretion was unaffected. Bioassay of the growth hormone for ACTH by Dr. V. K. Vance (63) demonstrated approximately 0.015 Units of activity in the daily dose.

DNA per adrenal gland in the growth hormone treated group, mean 3.0 mg., was significantly greater than in the control group ($P < 0.01$). RNA also was significantly greater ($P < 0.001$), 1.7 mg. than in the control group.

DISCUSSION

The synthesis of DNA as measured by incorporation of precursor molecules (64–66) and net increase in the DNA (67, 68) of the nuclear chromatin occurs in conjunction with cell division. DNA is essentially constant in amount per nucleus in non-proliferating cells (69, 70), its metabolism proceeding at a very slow rate (68, 71). Increase in the amount of mitotic activity would be reflected by increase in the total DNA of an organ and should indicate an increment in cell number. The RNA in cells is not homogenous and exhibits variation in metabolic rate partially dependent on its intracellular location (72–74). Most RNA in the cell is cytoplasmic, and the greatest amount is found in the "microsomes" (75, 76). "Microsomal" RNA has been shown to be involved in protein synthesis, probably in the assembly of activated precursors into protein macromolecules (77, 78).

In several systems, a close correlation between RNA content and protein synthesis has been observed (77, 79), and RNA synthesis has been related to induction of specific enzyme activity (81, 82). The rate of total RNA metabolism in terms of precursor incorporation (83–85) greatly exceeds that of DNA in non-proliferating cells; indeed, a rapid turnover of RNA precursors and net synthesis of RNA reflect metabolic activity (74).

Increase in RNA content of the adrenal cortex has been indicated histologically after infection and stress (86, 87) and after exogenous ACTH

administration (88–91). Symington *et al.* in their histological investigations of the human adrenal cortex (89, 92) found that in the "normal" gland most RNA is in the zona reticularis, while the zona fasciculata became "compact"; lipid depleted, rich in RNA, and increased in histochemical enzyme activity. With large doses of ACTH these changes involved both reticularis and fasciculata with apparent compression of the zona glomerulosa.

The RNA increase after ACTH in the present study is in agreement with the histological demonstrations, with the chemical determinations of RNA by Mandel *et al.* (93), Symington and Davidson (94), and Fiala *et al.* (95), and with the increased P^{32} labelling of RNA nucleotides reported by Logan *et al.* (96). The striking increase in canine adrenal RNA and the increase in gland weight and cortical width was accompanied by increase in urinary steroid metabolites (Table 3). The absence of significant DNA increase indicates that the number of cortical cells remained unaffected by ACTH administration and leads to the conclusion that ACTH increases adrenal weight through cell hypertrophy rather than through hyperplasia. Symington and Davidson found unchanged DNA-phosphorous (94) after ACTH; and histochemical studies (97, 98) of the DNA in individual adrenal cortical nuclei have indicated no change in the amount of DNA per cell after ACTH.

The increase in mitochondrial RNA and the even greater increase in "microsomal" RNA after ACTH administration, also indicated by Fiala *et al.* (99), probably corresponds to the increase in basophilic cytoplasmic granules described in histologic studies of ACTH effects. Mitochondria of the zona fasciculata and reticularis are increased in number by ACTH stimulation (100–103); they are the site of 11-β hydroxylation (104), an activity necessary for corticosteroid synthesis. Only minimal histological changes in the zona glomerulosa take place (50, 105). The general relation of microsomal RNA to protein synthesis in other tissues, and the increase in other enzyme activities observed histochemically after ACTH suggest that the ACTH-induced rise in microsomal RNA may be related to the synthesis of enzyme protein. Although it may be a long-term response to ACTH (95, 106), net protein synthesis has not been clearly demonstrated *in vitro* (107, 108). The correlation between cytoplasmic RNA and endocrine activity of the adrenal cortex seems reliable, but the specific functions of adrenocortical mitochondrial and microsomal RNA remain to be elucidated.

Compensatory hypertrophy of the remaining adrenal after unilateral adrenalectomy in the rat has been reported not to exceed a 70% increase of gland weight (43) and to be maximal between five and ten days (49). In this study the increases in weight were 45%, 39%, 13%, and 0%; the cortex, although thicker than normal, was not as thickened as with ACTH. RNA was not consistently elevated, a finding also reported by Villela and

Provoa (109); nor were urinary steroid levels consistently elevated in the adrenalectomized dogs (Table 3). If endogenous ACTH secretion had been specifically elevated in response to removal of the left adrenal rather than to post-operative stress of varying duration, RNA and urinary steroids should have been significantly elevated in all the dogs. This, however, was not observed (Table 3). DNA was elevated in all of the remaining right adrenals. The lack of difference in DNA content of the left and right glands of normal controls (Table 4) was in agreement with the report of Povoa and Fontana (110). Accelerated mitotic activity (44) and cell proliferation was therefore involved in this "compensatory hypertrophy" and appears to have been a primary response, for the increase in DNA did not correlate with either RNA content or gland weight.

Adrenal weight increase after growth hormone was, as expected, (22, 23, 111) less than the increase in canine adrenal weight after ACTH; and could be accounted for by increase in the size of cortex. DNA per gland was significantly increased after growth hormone administration; RNA was also increased, but less than after ACTH. The consistent urinary 17-KS and adrenal gland RNA elevations (Table 3) after growth hormone may have been a consequence of its administration—histochemical change in RNA in the zona glomerulosa has been reported (112)—but might be attributable to the demonstrated ACTH contamination of the growth hormone preparation. The DNA increase indicates that growth hormone has a stimulatory effect on cell proliferation. The evidence of the present study is supported by observations of increased mitotic activity in the zona fasciculata of rat adrenal cortices after growth hormone administration, by Stack-Dunne *et al.* (8, 21).

ACTH administration resulted in marked RNA increase in the canine adrenal gland and appears to have increased gland weight through cell hypertrophy rather than through hyperplasia, for DNA was not significantly affected. The adrenal cortex has a continuing low degree of cell division (21), the greatest mitotic activity being in the outer fasciculata (113). The mitotic rate, low under normal conditions, decreases after hypophysectomy and is only partially restored by ACTH, addition of growth hormone restoring it to normal. Increases in cortical mass or adrenal gland weight were much less with growth hormone (Table 3), (8) a potent stimulus to mitosis, than increases attainable with ACTH. Nevertheless, the location of mitotic figures in "compact," apparently RNA rich fasciculata cells (21, 44), the observations of increase in cytoplasmic elements after ACTH administration, and the theory that mitosis in a cell is inevitable when the cytoplasmic subcellular elements attain a critical mass (99, 114) suggest that ACTH stimulation could lead eventually to cell proliferation. Significant cell proliferation in response to ACTH seems unlikely, however, when the large increase in canine adrenal DNA after growth hormone is considered (Table 3). The lack of a change in gland DNA after seven days

of large amounts of bovine depot-ACTH also suggests that an increase in cytoplasmic elements does not exert a decisive influence in adrenal cortical mitosis, but definitive studies with species specific ACTH and long-term administration have not yet been undertaken.

The implication of growth hormone as a physiologically important corticotropic factor, is far from conclusive, and even if valid, may not preclude the existence of other factors. Nor does it exclude the possibility of increased target organ stimulation because of decreased levels of specific growth inhibiting substances (115).

This study presents evidence for the existence of adrenocorticotrophic influences that are not only separate from ACTH, but differ in their stimulation of RNA and DNA synthesis and cell proliferation. The similarity of nucleic acid changes after unilateral adrenalectomy and growth hormone administration raises the question of the possible chemical similarity of the adrenocortical stimuli involved.

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FACTORS INFLUENCING THE RELEASE OF FREE FATTY ACIDS FROM RAT ADIPOSE TISSUE[1]

R. M. BUCKLE, D. RUBINSTEIN, E. E. McGARRY[2] AND J. C. BECK

The McGill University Clinic, Royal Victoria Hospital and the Department of Biochemistry, McGill University

ABSTRACT

This report deals with some of the factors influencing the release of free fatty acids (FFA) from the rat epididymal fat pad. Glucose decreased the release of FFA by adipose tissue. This effect is seen to a lesser degree in tissue taken from untreated diabetic animals. Insulin does not appear to affect this activity regardless of the source of the adipose tissue. Pyruvate, fructose and galactose decreased the release of FFA by adipose tissue from normal animals. Fluoride completely prevents the action of glucose or pyruvate in inhibiting FFA release whereas iodoacetate prevents only the action of pyruvate. Dinitrophenol has no effect on glucose or pyruvate inhibition of FFA release. This suggests that glucose and pyruvate exert their action by providing precursors of glycerol phosphate needed for re-esterification of the FFA. Glucose inhibits the release of FFA induced by growth hormone, thyrotropin and ACTH, but has no effect upon the release induced by epinephrine.

THE role of lipids in tissue metabolism has been the subject of extensive investigation in recent years (1). *In vivo* the level of plasma free fatty acids (FFA) is influenced by a variety of agents. Hormones such as epinephrine (2), growth hormone (GH) (3) and ACTH (4) have been shown to increase, while ingestion of carbohydrate and administration of insulin to decrease the concentration of FFA in the plasma (5).

Most of these effects can be duplicated *in vitro* using the rat epididymal fat pad. Release of FFA has been reported when this tissue is incubated with GH, ACTH, adrenalin (6) and TSH (7). On the other hand the addition of glucose and insulin to the incubation medium inhibits release of FFA and, in the presence of large amounts of FFA, promotes uptake and esterification of FFA by the tissue (5, 9). This report deals with experiments performed in an attempt to assess the role of glucose and a number of hormones in the release of FFA from adipose tissue *in vitro*.

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[2] Research Associate, National Research Council, Canada.

MATERIALS AND METHODS

Male hooded rats weighing 190 to 210 gm. were fasted for 24 to 48 hr. The animals were anaesthetized with Nembutal and the epididymal fat pads removed and weighed. In the earlier experiments intact fat pads were incubated while in subsequent studies each pad was longitudinally sectioned and weighed prior to incubation. The adipose tissue was placed in 4 ml. of a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% rabbit albumin and equilibrated at 37° C with an atmosphere of 95% O_2, 5% CO_2 for ten minutes after which a sample of the medium was taken for FFA determination. A second sample was withdrawn after an additional incubation of two hours. FFA was determined by the method of Dole (2). The results were expressed as micromols of FFA released per gram of wet weight of tissue per hour.

The FFA release from intact and hemi-pads in some typical control experiments is shown in Table 1. It can be seen that FFA release from fragments of adipose tissue obtained from the same animal is comparable, but the values obtained from different animals are variable. Thus, in most of the following tables the control FFA release is taken as 1.00 and the experimental release compared with this figure for each separate animal.

TABLE 1. COMPARISON OF FFA RELEASE FROM EPIDIDYMAL FAT PADS

Animal	Pad	FFA release (μM FFA gm. wet wt./hr.)					
		Side A		Side B		Mean	S.D.
1	intact	0.55		0.51		0.53	
2	intact	1.77		1.73		1.75	
3	intact	2.31		2.37		2.34	
4	halved	1.00	1.04	0.95	1.02	1.00	±0.04
5	halved	1.97	1.75	1.82	1.68	1.80	±0.12
6	halved	2.25	2.12	2.23	2.12	2.18	±0.07
7	halved	2.34	2.29	2.25	2.26	2.29	±0.04

The FFA content of adipose tissue was determined by rinsing the tissue in ice cold Krebs-Ringer buffer, homogenizing in the extraction mixture recommended by Dole (2) and titrating as for the estimation of FFA in the incubation medium. Glucose was estimated by the method of Nelson (8).

Diabetic rats were prepared by injecting 15 mg. Alloxan per 100 gm. body weight intraperitoneally. The animals were untreated and not used until they showed ketonuria, glycosuria and a blood glucose level of over 250 mg. %.

RESULTS

The Effect of Glucose and Insulin on FFA Release

The effect of glucose and insulin upon the spontaneous release of FFA by the rat epididymal fat pad was studied by incubating the pad or hemi-pad with these agents either separately or combined. The results are seen in Table 2.

The data confirm previous observations that glucose and insulin cause a marked decrease in the release of FFA. However, this effect could readily be produced by glucose in the absence of insulin. Fasting the animals for 72 hours, at which time endogenous insulin in the tissue should be minimal, failed to influence the action of glucose. In several experiments the insulin content of the flasks was increased 10-fold but this likewise did not influ-

TABLE 2. EFFECT OF GLUCOSE AND INSULIN UPON THE RELEASE OF FFA. RELATIVE RELEASE OF FFA (CONTROL = 1.00)

Preparation used	Hours fasted	Glucose added 100 mg. %	Insulin (10 milliunits/ml. medium added)	Glucose and Insulin added
Intact pads	24	0.23 ±0.05* (10)	1.15 ±0.21 (6)	0.33 ±0.06 (12)
Half pad	36	0.17 ±0.02 (5)	1.14 ±0.15 (5)	0.14 ±0.18 (5)
Half pad	48	−0.10** (2)	1.42 (2)	0.13 (2)
Half pad	72	0.13 (2)	—	—

* Standard error of mean.
Figures in parentheses represent number of experiments.
** Represents uptake of FFA from medium.

ence the results. Insulin retained its ability to stimulate increased glucose uptake in these experiments. The decrease in FFA release into the medium was paralleled by a decrease in tissue FFA content as is demonstrated in Table 3. It will be noted that insulin did not enhance the effect of glucose on tissue FFA.

The role of glucose and insulin in the release of FFA by adipose tissue from alloxan diabetic animals was also studied (Table 4). The data indicate that in this tissue the presence of glucose also results in a significant decrease in FFA release. This release is not further influenced by the addition of insulin to the medium. However, the effectiveness of glucose in inhibiting FFA release appears to be progressively diminished as the severity of the diabetic state is increased. Thus glucose produced the smallest inhibition of FFA release from the adipose tissue of the most severely diabetic animals.

TABLE 3. COMPARISON OF FFA CONTENT OF ADIPOSE TISSUE AND MEDIUM AFTER INCUBATION WITH GLUCOSE AND INSULIN

Flask contents	Animal									
	1		2		3		4		5	
	Tissue*	Medium**	Tissue*	Medium**	Tissue*	Medium**	Tissue*	Medium**	Tissue*	Medium**
Control	5.25	1.84	8.39	4.11	2.19	2.11	3.62	3.10	2.95	1.28
Glucose (100 mg. %)	1.84	0.28	1.74	0.71	1.18	0.0	2.12	0.85	1.11	0.35
Insulin (10 milliunits per ml.)	5.19	2.34	9.51	5.49	—	—	—	—	2.54	1.26
Glucose +Insulin	1.60	0.51	3.10	1.22	1.16	−0.82***	1.83	0.55	—	—

* uM FFA/gm. wet weight of tissue at end of incubation.
** uM FFA released into medium/gm. wet weight of tissue.
*** Represents uptake of FFA from medium.

TABLE 4. RELEASE OF FFA FROM ADIPOSE TISSUE OF DIABETIC RATS

Blood sugar range (mg. %)	Relative release of FFA (control = 1.00)		
	Glucose added 100 mg. %	Insulin added 10 milliunits/ml.*	Glucose and insulin added
270–440	0.41 ±0.09** (8)	1.03 ±0.07 (5)	0.41 ±0.12 (7)
650–910	0.67 ±0.06 (6)	1.10 ±0.18 (5)	0.78 ±0.12 (6)

* In a number of experiments 20–40 milliunits/ml. of insulin were used. This did not alter the results.
** Standard error of the mean.
Figures in parentheses represent the number of experiments.

TABLE 5. EFFECTIVENESS OF GLUCOSE, FRUCTOSE AND PYRUVATE IN INHIBITING FFA MOBILIZATION *in vitro*

Concentration mg./100 ml. medium	Relative release of FFA (Control = 1.00)			
	Glucose	Fructose	Galactose	Pyruvate
100	0.23	0.45	0.45	0.15
50	0.22	0.92	0.69	0.13
25	0.39	1.10	1.05	0.24
12.5	0.52	—	—	0.42
6.2	0.91	—	—	0.62

The effect of glucose, fructose, galactose, pyruvate, sucrose and ribose on the net release of FFA from the rat fat pad was investigated. Sucrose and ribose caused no interference with the release of FFA from the fat pad. Typical results obtained with glucose, fructose, galactose and pyruvate are shown in Table 5. Concentrations of both glucose and pyruvate down to 12 mg. % were effective in decreasing the net amount of FFA found in the medium after incubation. On the other hand much higher concentrations of fructose and galactose were needed to bring about this effect, a finding which is consistent with the rate at which these carbohydrates usually are metabolized as compared with glucose.

In view of the metabolic implications of the action of glucose and pyruvate in depressing FFA release, several experiments were carried out to study the action of various metabolic inhibitors such as 2,4,dinitrolphenol (DNP), iodoacetate and fluoride. The results are shown in Table 6. DNP, which uncouples oxidative phosphorylation, has no effect on the release of FFA from the tissues in the absence of substrate. Similarly, it in no way affects the decrease in release noted with glucose or pyruvate.

The addition of fluoride to the incubation medium increased the release of FFA in the absence of substrate. This action was only slightly decreased by the addition of glucose. On the other hand, in the presence of pyruvate the release of FFA was increased even further.

The addition of 4×10^{-3}M iodoacetate, unlike fluoride, does not influence the release of FFA in the absence of substrate. However, it reduces the inhibitory influence of glucose but does not prevent it entirely. On the other hand, in the presence of iodoacetate, pyruvate is completely ineffective in

TABLE 6. THE EFFECT OF VARIOUS INHIBITORS ON THE RELEASE OF FFA

Inhibitor	Concentration of inhibitor	Relative FFA release (control = 1.00)		
		No substrate	Glucose added 100 mg. %	Pyruvate added 100 mg. %
None	—	1.00	0.11 ± 0.05 (19)	0.18 ± 0.05 (18)
Dinitrophenol	5×10^{-4}M	0.95 ± 0.07* (11)	0.07 ± 0.05 (9)	0.19 ± 0.09 (5)
Fluoride	4×10^{-2}M	1.39 ± 0.10 (5)	1.25 ± 0.10 (6)	1.69 ± 0.10 (6)
Iodoacetate	4×10^{-3}M	1.02 ± 0.05 (13)	0.57 ± 0.10 (7)	1.25 ± 0.08 (7)
Iodoacetate	4×10^{-4}M	—	0.11 ± 0.05 (8)	1.09 ± 0.12 (9)

* Standard error of the mean.
Figures in parentheses represent number of experiments.

preventing a net release of FFA. When the concentration of iodoacetate is decreased to 4×10^{-4}M there is no interference with the action of glucose in diminishing FFA release, but the decrease in FFA attributable to pyruvate is still completely inhibited. A few experiments, not reported here, were also carried out under anerobic conditions in the absence of any inhibitor. No influence on the action of glucose or pyruvate was noted under these conditions.

The Effect of Glucose on the Stimulation of FFA Release by Various Hormones

Glucose inhibits at least partially the release of FFA which is stimulated by ACTH, GH and TSH. However, it appears to have no effect on the

TABLE 7. THE EFFECT OF GLUCOSE IN REDUCING HORMONE STIMULATED FFA RELEASE

Hormone	μg/ml.	Relative FFA release* (control = 1.00)		
		+Hormone	+Glucose	Glucose and Hormone
Adrenalin	0.1	3.2	0.16	3.1
	2.0	5.8	0.41	6.2
ACTH	0.01	1.7	0.04	0.13
	0.10	4.5	0.11	3.4
STH	5.0	3.7	−0.32**	2.1
	30.0	6.1	0.70	4.1
TSH	0.5	1.6	0.39	0.45
	3.0	1.9	0.51	0.76

* Each value is the average of 4 separate experiments.
** Represents uptake of FFA from the medium.

release of FFA induced by epinephrine. Table 7 illustrates some typical experiments of this type. The efficacy with which glucose prevents the release of FFA probably depends on the amount of hormone added to the medium.

DISCUSSION

It is generally accepted that the mobilization of tissue FFA is a reflection of lipolysis of triglyceride in the adipose tissue. Thus, changes causing an *in vivo* increase in the plasma FFA are paralleled by increases of mono and diglycerides in the adipose tissue (11). A comparable production of FFA has been demonstrated *in vitro*. However, diffusion into the medium occurs only in the presence of an acceptor such as albumin (12). Simultaneous measurements of tissue and medium FFA have shown changes in the two values to parallel each other in the presence of albumin (9, 10), an observation which has been confirmed in this paper.

The finding that a decrease in FFA release could be obtained with glucose either in the presence or absence of insulin is in accord with the observations of Cahill, *et al.* (13) that glucose alone stimulates the uptake of palmitate-C^{14}. Bally *et al.* (14) have shown that only a slight increase in

palmitate uptake occurs in the presence of large amounts of insulin. Similarly Bierman *et al.* (15) using totally pancreatectomized dogs concluded that insulin was not required for the uptake of FFA. It is improbable that the effects attributed to glucose in the present study are due to the presence of a residual amount of endogenous insulin, since glucose was shown to be effective in inhibiting FFA release by tissue obtained from diabetic animals. It is of interest that adipose tissue from diabetic rats is much less sensitive to the inhibition of FFA release by glucose and this inhibition is unaltered by insulin. This is most noticeable in tissues taken from the severely diabetic rats using the blood sugar level as a criterion. This may bear a relationship to the observation that in spite of hyperglycemia an elevated plasma FFA concentration occurs in the uncontrolled human diabetic. A possible mechanism may involve a greater rate of lipolysis. This suggestion is supported by the observation that a higher control FFA release was obtained from the pads of the more severely diabetic animals.

There are several mechanisms by which glucose may exert its effect. White (6) has suggested that glucose inhibits lipolysis. On the other hand, glucose may increase triglyceride synthesis by one of the following mechanisms. (1) It may supply the energy required for the formation of acyl-CoA derivatives prior to esterification of the fatty acid with glycerol phosphate. (2) It may provide the precursor necessary for glycerol phosphate synthesis. The ability of glucose, pyruvate, and to a lesser extent fructose and galactose to produce inhibition of FFA release suggests that these substances play a role in the synthesis of triglycerides. The inhibition of FFA release resulting from the presence of glucose and pyruvate is completely antagonized by fluoride. This is consistent with the observation that fluoride inhibits the esterification of glycerol phosphate (19). The possible increase in FFA release in the presence of fluoride may be due to lipogenesis from pyruvate. The inhibition of enolase activity by fluoride precludes a similar lipogenesis from glucose.

The possibility that the substrates studied here promote esterification by supplying the necessary energy may be excluded by the observations with DNP. This substance, which uncouples oxidative phosphorylation, had no effect on FFA release. Robell (16) has found that DNP interferes with FFA uptake, but under his conditions a large excess of FFA was added to the medium, in which case there may have been an insufficient concentration of endogenous ATP to supply the needs of the esterifying system.

The decrease in FFA release in the presence of glucose was not affected by 4×10^{-4}M iodacetate while the pyruvate induced inhibition was completely nullified. In the course of glycolysis glucose is broken down to triose phosphate. Similarly through a reversal of glycolysis pyruvate may serve as a precursor of triose phosphate. In the latter sequence the terminal reaction is catalyzed by triose phosphate dehydrogenase which is inhibited by iodoacetate. The triose phosphate formed by either pathway may then be reduced to glycerol phosphate. Our observations with metabolic inhibi-

tors therefore lead to the conclusion that glucose and pyruvate increase esterification of FFA by supplying the glycerol moiety of the triglycerides. This data supports the observations of Shapiro *et al.* (17) and Cahill *et al.* (18) that the limiting substrate in triglyceride synthesis is the supply of glycerol phosphate, since the adipose tissue cannot phosphorylate free glycerol.

Insulin has a relatively slight stimulatory action on glyceride-glycerol synthesis (2- to 3-fold) from glucose as compared with fatty acid and glycogen formation (50-fold) (18). The observation that insulin does not stimulate FFA esterification is consistent with the conclusion that glucose acts primarily as a precursor of glycerol phosphate.

It is generally believed that various hormones such as G H, TSH and ACTH, cause an increase in lipolysis (20, 21). The action of glucose under these circumstances counteracts that of the hormones by re-esterifying the FFA released by the increased lipolysis. The failure of glucose to reverse the action of adrenalin is presently under study.

Addendum

Since submission of this manuscript Freinkel (*J. Clin. Invest.* **40:** 476. 1961) has reported an *in vitro* effect of TSH on FFA release.

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FOLLICULAR GROWTH AND OVULATION IN HYPOPHYSECTOMIZED HENS

H. OPEL[1] AND A. V. NALBANDOV[2]

Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT

Growth of ovarian follicles was induced in hypophysectomized laying hens by daily intramuscular injection of mammalian FSH, PMS or chicken anterior pituitary powder (CAP). The mammalian gonadotropins caused a pronounced increase in the number of follicles entering the initial phase of yellow yolk deposition, but did not support growth of follicles to mature size. The disproportional accumulation of small follicles resulted in the complete obliteration of the normal follicular hierarchy. CAP produced no overstimulation of the smaller follicles and, in a significant number of birds, established a follicular order similar to that found in intact hens. In some CAP treated hens follicles developed to mature size by the end of the eight day experimental period.

With a timed sequence of injection of ovulation-inducing substances up to five consecutive daily ovulations were obtained. The number of ovulations was limited by the ability of follicles to attain ovulable size and was independent of the type of gonadotropin used to stimulate follocular growth.

OVARIAN function in the laying hen depends upon the presence and the continued maintenance of a hierarchy of follicles of graded sizes only one of which matures and ovulates at any one time.

Evidence for the role of gonadotropins in follicular growth has been derived largely from the administration of these hormones to intact chickens in varying reproductive condition. In immature birds precocious growth of follicles cannot be obtained with gonadotropins of mammalian origin (1, 2), and, while extracts of whole avian pituitaries cause some follicles to enter the phase of rapid growth, a normal follicular hierarchy is not established and no follicles reach maturity (1, 3). In intact laying hens repeated injections of mammalian preparations result in excessive stimulation of smaller follicles, leading to obliteration of normal size gradation and cessation of ovulation (4, 5). As many as eight follicles may be cased to reach ovulatory size and these can be made to ovulate in response to a single injection of a mammalian luteinizing hormone (LH) preparation. Experiments of this type demonstrate that gonadotropins cause follicular

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[1] Present Address: Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

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growth and suggest qualitative differences between mammalian and avian hormones.

MATERIALS AND METHODS

Columbian-New Hampshire crossbred hens were used in preliminary trials and White Leghorn hens in the third to sixth months after onset of lay were used in the final experiments. Hens were kept in individual laying cages, were fed a standard ration and were exposed to a constant 14-hour light-day (6 A.M. to 8 P.M.). Records of lay were maintained for two weeks prior to and during the whole experimental period. Only those hens laying four or more eggs in sequence (clutch), with a pause of no more than one day between sequences, were selected for study.

Following the removal of the anterior lobe of the pituitary by the transbuccal approach (1) the birds were placed in individual cages in a constant temperature room at 78° F. In addition to the standard 14-hour light-day, light was provided from 9 to 11 P.M. and from 1 to 3 A.M. in order to prevent nocturnal fasting and thus to keep postoperative mortality to a minimum (7).

Mammalian gonadotropins were purified preparations of a follicle stimulating hormone (FSH) (Armour, Lot R 377201), luteinizing hormone (LH) (Armour, Lot R 377279) and pregnant mare serum (PMS) (Upjohn, Lot KS 604 FH). Assay of the FSH and LH preparations on rats showed that neither preparation was pure and that they can be best characterized as "predominantly" FSH or LH. According to the manufacturer, the FSH contained about 5% LH on a dry matter basis, while the LH contained "some" prolactin and thyrotropin and (as will be shown later) significant amounts of FSH. The LH-contaminant in FSH was sufficient to cause comb growth in hypophysectomized hens if FSH was given in sufficiently high doses. The hormones were dissolved in physiological saline at concentrations permitting the use of a standard volume of 0.2 cc. per injection. Avian gonadotropins were given in the form of a whole, air dried, chicken anterior pituitary powder (CAP), containing all the hormones usually expected in such a powder, and prepared from pooled glands of chickens of both sexes varying in age from $2\frac{1}{2}$ to $3\frac{1}{2}$ months. Immediately before use the powder was suspended in saline at a concentration of 4 mg. per cc.

Hormones used for ovarian maintenance were administered by single daily intramuscular injection while ovulations were induced by injection of hormones into the wing vein.

Hypophysectomized chickens not treated with hormones and kept in proper environmental conditions survive without difficulty for weeks or months. Treatment with gonadotropic hormones forces them to mobilize about 14% of their body mass for the formation of yolk or for the formation of the completed egg which they were induced to lay. Under the condition of this experiment, hens survived for only about 12 days after hypophysectomy and hormone treatment. Guided by these observations made in preliminary experiments, it was decided to kill hens 2, 4, 6 or 8 days after hypophysectomy. The decision to kill a bird on a particular day sometimes depended on an estimate of its chance for survival until the next set interval. Moribundity was usually signaled by cyanosis of the combs occurring several hours before death. Birds which died between scheduled times were excluded from the data reported.

Body weight and bomb area (length×height) were obtained at the beginning and end of each study. Changes in comb size are expressed as percent of the original comb area. At autopsy the ovaries and oviducts were weighed, each intact follicle was removed and weighed and was assigned to one of five arbitrary size classes. The number of atretic follicles was also recorded.

Ovulations were detected by digital palpation of the oviduct for the presence of ova and were confirmed by the laying of the completed egg.

The yolks of the first and second eggs of a clutch laid by hens destined for hypophysectomy were weighed for comparison with the weights of ova obtained by forced ovulation after hypophysectomy.

RESULTS

Preliminary trials to establish hormone levels and schedule of injections

Optimal levels of each hormone were chosen from a range of dosages used in extensive preliminary trials in which combinations of FSH and LH or TSH were used. The criterion for the optimal dose was its ability to maintain normal ovarian size and function for 8 days without destroying the hierarchy, without allowing follicular atresia, and without causing marked deviations in the weight of the oviduct or the size of the comb. Daily injections of 2.0 I.U. of FSH, or of 12.0 Cartland-Nelson Units (CNU) of PMS, or of 4.0 mg. of CAP came closest to fulfilling these specifications and were used in all experiments. A single intravenous injection of 2.0 mg. of LH or of 0.5 mg. of CAP consistently caused ovulations.

A comparison of effects between treatments required that the forced sequence of ovulations be initiated in each hen at a comparable point in the natural cycle. It was essential to remove the pituitary prior to the normal episodic release of LH which would lead to rupture of the mature follicle and which may occur as early as 14 hours before ovulation (8). Since the hens used were laying clutches of 4 to 20 or more eggs, it was difficult to predict the end of a clutch from past records of lay. It was decided to allow lay of the first egg (called C1), followed by the ovulation of the second follicle (C2) of a clutch and to hypophysectomize each hen 18 hours prior to the estimated time of ovulation of the third (C3) follicle. Hormones for the support of ovarian function were injected at the time of operation and at 24 hour intervals thereafter. Ovulation-inducing hormones (OIH) (which could be either LH or CAP) were injected 11 hours before estimated C3 ovulation and injections were repeated at 24 hour intervals. The time of 11 hours was selected as the midpoint of the probable period of normal LH-release which was estimated by van Tienhoven *et al.* (7) to occur 8 to 14 hours prior to ovulation.

Comparison of the effects of FSH, PMS and CAP on the maintenance of ovarian function

A total of 102 hypophysectomized hens was assigned at random to treatments with FSH, PMS or CAP. The effects of the three treatments (Table 1) were compared by calculating the linear regression (method of least squares) for the rates of change in the weights of the ovaries, total weight of follicles and oviducts, and in comb areas. Total follicle weight, which reflects the quantity of deposited yolk, showed a significant trend to increase

TABLE 1. WEIGHT OF OVARY, FOLLICLES AND OVIDUCT, COMB SIZE, AND NUMBER OF ATRETIC FOLLICLES IN HYPOPHYSECTOMIZED HENS RECEIVING GONADOTROPINS FOR 2 TO 8 DAYS

Treatment	Days	No. of hens	Ovary weight (gms.)	Total Follicle weight (gm.)	Oviduct (gm.)	Comb % change	Av. no. atretic follicles
Control	0	15	49.9	43.5	49.2	—	0.07
FSH	2	11	35.1	25.6	49.0	−1.1	0.50
	4	10	39.5	30.5	45.3	+1.2	0.50
	6	5	49.2	42.8	54.2	−2.5	0.20
	8	4	101.5	88.5	48.0	−6.6	1.50
PMS	2	8	34.8	28.8	45.6	−0.7	0.13
	4	10	39.3	30.6	47.7	+0.4	1.00
	6	5	48.2	38.0	51.2	+1.7	0.60
	8	4	75.2	61.3	60.5	+4.9	1.50
CAP	2	8	33.0	23.0	46.0	−0.4	0.25
	4	8	40.0	31.0	48.6	−5.2	0.25
	6	8	58.4	48.9	45.5	+0.6	1.00
	8	8	56.3	45.8	46.4	+1.1	2.80

with increasing time after hypophysectomy. The rate of increase differed significantly ($P < 0.05$) with treatment, being greatest in the hens given FSH ($b = 10.04$), least in hens receiving CAP ($b = 4.38$) and intermediate for hens treated with PMS ($b = 5.23$). The changes in oviduct size were not significant. Comb area in hens injected with PMS or CAP exhibited a significant trend to increase, while comb area in FSH treated birds decreased with time.

The distribution of atretic follicles was apparently heterogeneous (Table 1). Atresia was not confined to any particular follicle size and was limited to only a few birds. The increased incidence of atresia noted on day 8 was due to extensive follicular degeneration in one or two hens in each group.

Follicular growth and the hierarchy

The progressive changes in the follicular hierarchy seen in hens given FSH, PMS or CAP are presented in Table 2. FSH caused a pronounced overstimulation of the smaller follicles and a three-fold increase by Day 8 in the total number of follicles entering the phase of rapid growth. The maximum size attained by the stimulated follicles was six to nine grams. PMS also produced an increase in the population of smaller follicles, but the size attained was not as great as that seen in FSH-treated birds and only a few of the follicles reached a weight of 8 to 10 grams. The disappearance of the larger follicles from the hierarchy, in nearly all cases, was due to ovulations without replacement from the lower echelons.

Multivariate analysis (method of least squares) of the changes occurring in the number of follicles found in certain arbitrary size classes in relation to time, showed a marked linear trend in the distribution of follicles in the

TABLE 2. MEAN NUMBER OF FOLLICLES IN SIZE CLASSES IN HYPOPHYSECTOMIZED HENS RECEIVING GONADOTROPIN

Treatment	Days	No. of hens	Size class of follicles (gms.) 0.09 to 0.99	1.0 to 2.9	3.0 to 5.9	6.0 to 8.9	9.0 to 11.9	12.0 to 15.0	15+	Total follicles
Control	0	15	8.0	0.9	1.1	0.9	0.9	0.8	0.4	14.1
FSH	2	11	17.1	1.6	0.7	0.4	0.9	0.0	0.1	20.9
	4	10	13.8	9.7	1.0	1.0	0.2	0.0	0.0	26.4
	6	5	18.0	11.8	0.8	0.4	1.0	0.0	0.0	32.4
	8	4	20.6	17.3	10.1	0.3	0.0	0.0	0.0	49.8
PMS	2	8	15.5	0.9	1.0	0.5	0.9	0.5	0.0	19.3
	4	10	17.5	2.5	0.6	0.5	0.8	0.5	0.0	22.8
	6	5	16.4	8.8	1.2	0.8	0.2	0.2	0.0	27.6
	8	4	19.6	1.1	5.0	0.8	0.3	0.0	0.0	36.8
CAP	2	8	11.3	1.3	0.9	0.9	0.4	0.1	0.1	15.2
	4	8	10.6	3.6	1.0	0.6	1.1	0.1	0.0	17.5
	6	8	8.8	5.0	5.5	1.1	0.5	0.1	0.0	21.6
	8	8	8.4	2.6	1.9	2.6	0.5	0.1	0.0	17.6

size categories, a trend which was clearly different in the treatments used (Table 3).

The array of follicles in hens given CAP differed from that produced by mammalian gonadotropins in three important respects: (1) There was no significant increase by Day 8 in the total number of rapidly growing follicles in the ovaries of the CAP treated hens. (2) CAP caused no accumulation of follicles in the initial stages of rapid growth (weighing 0.09 to 0.99 gm.). (3) In one-half of the CAP injected hens surviving to the end of the experimental period, one or more follicles developed to ovulable size and were ovulated. Four of the hens ovulated in response to the seventh or eighth OIH injection, while no ovulations were forced by these injections in hens given mammalian gonadotropins.

The types of follicular gradation produced by FSH and CAP are portrayed in Figures 1 to 16. One of the most striking features of the follicular hierarchy in hens given CAP was the extreme variability in the distribution of follicles in the ovaries of birds killed on days 6 and 8. In 4 of the 16 hens killed at these intervals, the follicular gradation was very similar to that

TABLE 3. ANALYSIS OF THE MEAN DAILY CHANGE IN THE DISTRIBUTION OF FOLLICLES WITHIN THE VARIOUS SIZE CLASSES

Treatment	Size class of follicles (gms.) 0.09 to 0.29	0.30 to 0.99	1.0 to 2.9	3.0 to 5.9	9.0 to 15+	Total no. of follicles
FSH	+0.11	+0.65	+2.48**	+1.46**	−0.14*	+4.56**
PMS	−1.00**	+1.57**	+1.74**	+0.69*	−0.21**	+2.79**
CAP	−0.22	−0.33	+0.36	+0.77**	−0.02	+0.56

Significantly different from controls *P < .05
**P < .01.

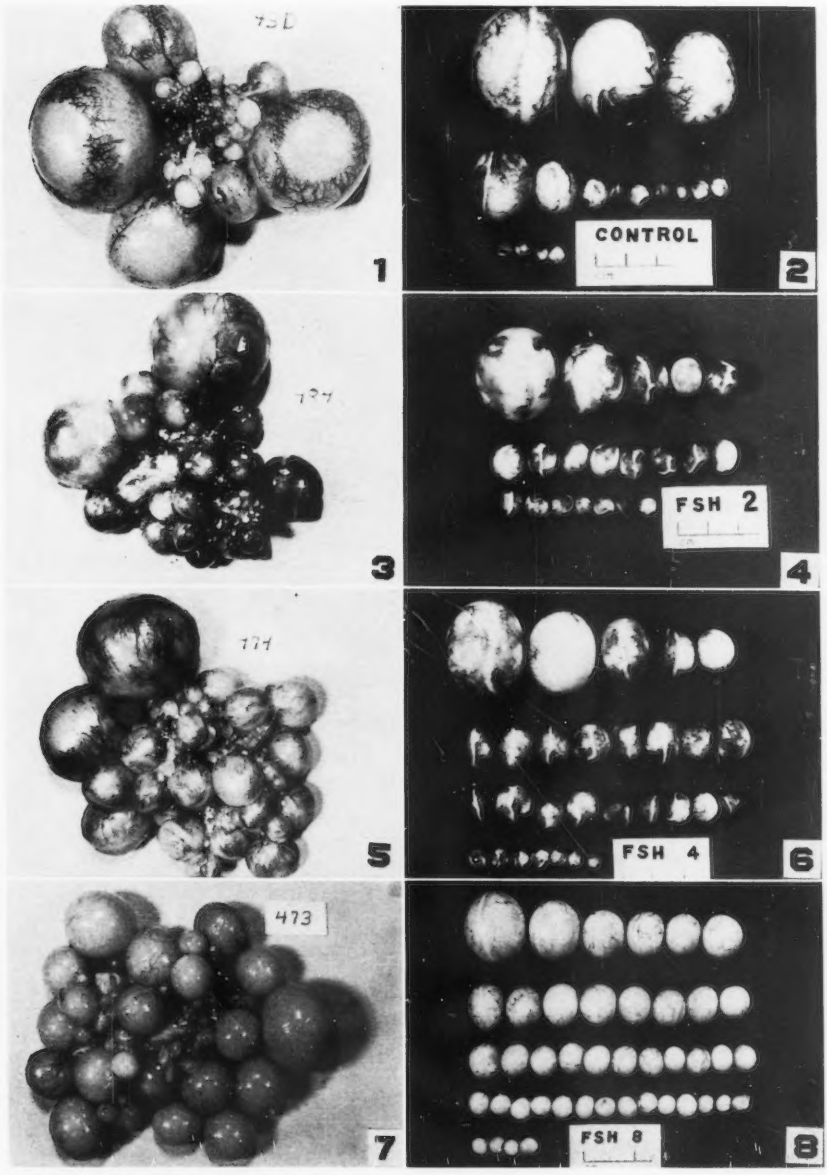

FIG. 1. Ovary of normal laying hen. Figs. 3, 5, 7. Ovaries of hypophysectomized hens treated with FSH for 2, 4 or 8 days. Figs. 2, 4, 6, 8. Hierarchies of follicles from ovaries shown in Figs. 1, 3, 5 and 7.

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	6	5	49.2	42.8	54.2	−2.5	0.20
	8	4	101.5	88.5	48.0	−6.6	1.50
PMS	2	8	34.8	28.8	45.6	−0.7	0.13
	4	10	39.3	30.6	47.7	+0.4	1.00
	6	5	48.2	38.0	51.2	+1.7	0.60
	8	4	75.2	61.3	60.5	+4.9	1.50
CAP	2	8	33.0	23.0	46.0	−0.4	0.25
	4	8	40.0	31.0	48.6	−5.2	0.25
	6	8	58.4	48.9	45.5	+0.6	1.00
	8	8	56.3	45.8	46.4	+1.1	2.80

with increasing time after hypophysectomy. The rate of increase differed significantly ($P < 0.05$) with treatment, being greatest in the hens given FSH ($b = 10.04$), least in hens receiving CAP ($b = 4.38$) and intermediate for hens treated with PMS ($b = 5.23$). The changes in oviduct size were not significant. Comb area in hens injected with PMS or CAP exhibited a significant trend to increase, while comb area in FSH treated birds decreased with time.

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TABLE 2. MEAN NUMBER OF FOLLICLES IN SIZE CLASSES IN HYPOPHYSECTOMIZED HENS RECEIVING GONADOTROPIN

Treatment	Days	No. of hens	Size class of follicles (gms.)							Total follicles
			0.09 to 0.99	1.0 to 2.9	3.0 to 5.9	6.0 to 8.9	9.0 to 11.9	12.0 to 15.0	15+	
Control	0	15	8.0	0.9	1.1	0.9	0.9	0.8	0.4	14.1
FSH	2	11	17.1	1.6	0.7	0.4	0.9	0.0	0.1	20.9
	4	10	13.8	9.7	1.0	1.0	0.2	0.0	0.0	26.4
	6	5	18.0	11.8	0.8	0.4	1.0	0.0	0.0	32.4
	8	4	20.6	17.3	10.1	0.3	0.0	0.0	0.0	49.8
PMS	2	8	15.5	0.9	1.0	0.5	0.9	0.5	0.0	19.3
	4	10	17.5	2.5	0.6	0.5	0.8	0.5	0.0	22.8
	6	5	16.4	8.8	1.2	0.8	0.2	0.2	0.0	27.6
	8	4	19.6	1.1	5.0	0.8	0.3	0.0	0.0	36.8
CAP	2	8	11.3	1.3	0.9	0.9	0.4	0.1	0.1	15.2
	4	8	10.6	3.6	1.0	0.6	1.1	0.1	0.0	17.5
	6	8	8.8	5.0	5.5	1.1	0.5	0.1	0.0	21.6
	8	8	8.4	2.6	1.9	2.6	0.5	0.1	0.0	17.6

size categories, a trend which was clearly different in the treatments used (Table 3).

The array of follicles in hens given CAP differed from that produced by mammalian gonadotropins in three important respects: (1) There was no significant increase by Day 8 in the total number of rapidly growing follicles in the ovaries of the CAP treated hens. (2) CAP caused no accumulation of follicles in the initial stages of rapid growth (weighing 0.09 to 0.99 gm.). (3) In one-half of the CAP injected hens surviving to the end of the experimental period, one or more follicles developed to ovulable size and were ovulated. Four of the hens ovulated in response to the seventh or eighth OIH injection, while no ovulations were forced by these injections in hens given mammalian gonadotropins.

The types of follicular gradation produced by FSH and CAP are portrayed in Figures 1 to 16. One of the most striking features of the follicular hierarchy in hens given CAP was the extreme variability in the distribution of follicles in the ovaries of birds killed on days 6 and 8. In 4 of the 16 hens killed at these intervals, the follicular gradation was very similar to that

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Treatment	Size class of follicles (gms.)					Total no. of follicles
	0.09 to 0.29	0.30 to 0.99	1.0 to 2.9	3.0 to 5.9	9.0 to 15+	
FSH	+0.11	+0.65	+2.48**	+1.46**	−0.14*	+4.56**
PMS	−1.00**	+1.57**	+1.74**	+0.69*	−0.21**	+2.79**
CAP	−0.22	−0.33	+0.36	+0.77**	−0.02	+0.56

Significantly different from controls *P < .05
**P < .01.

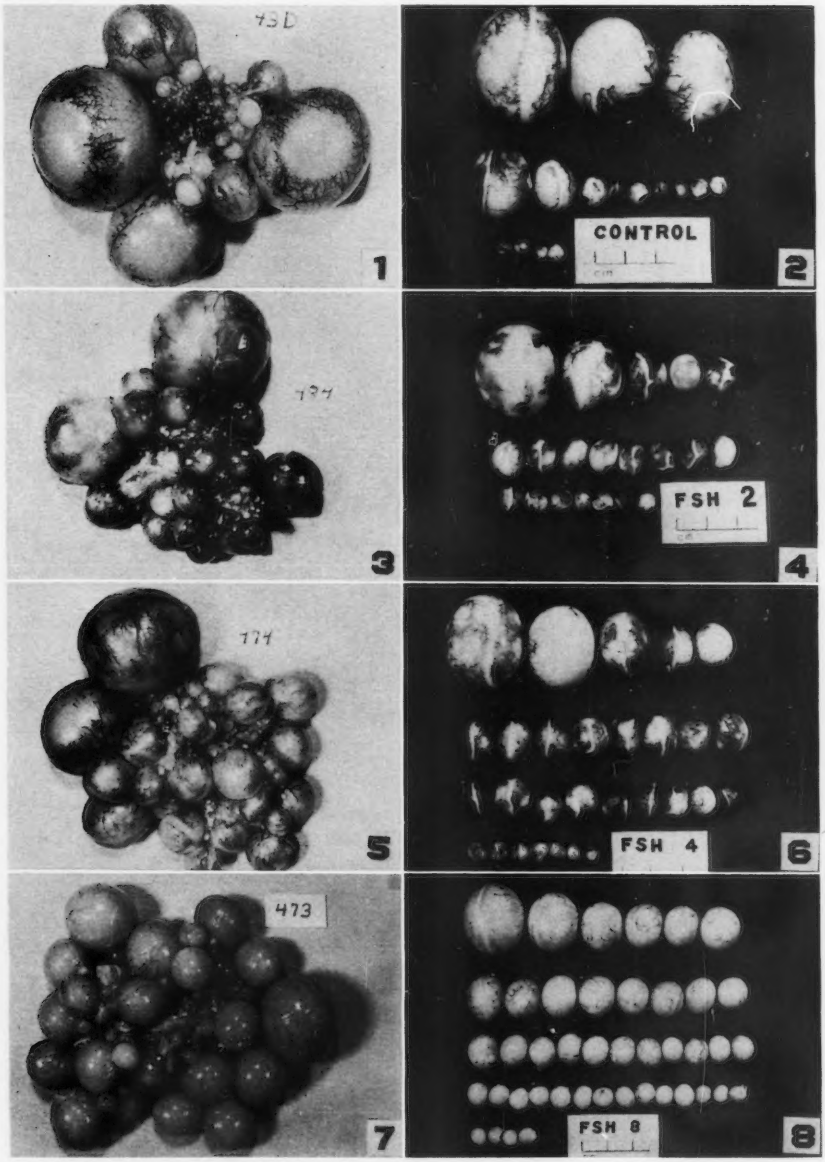
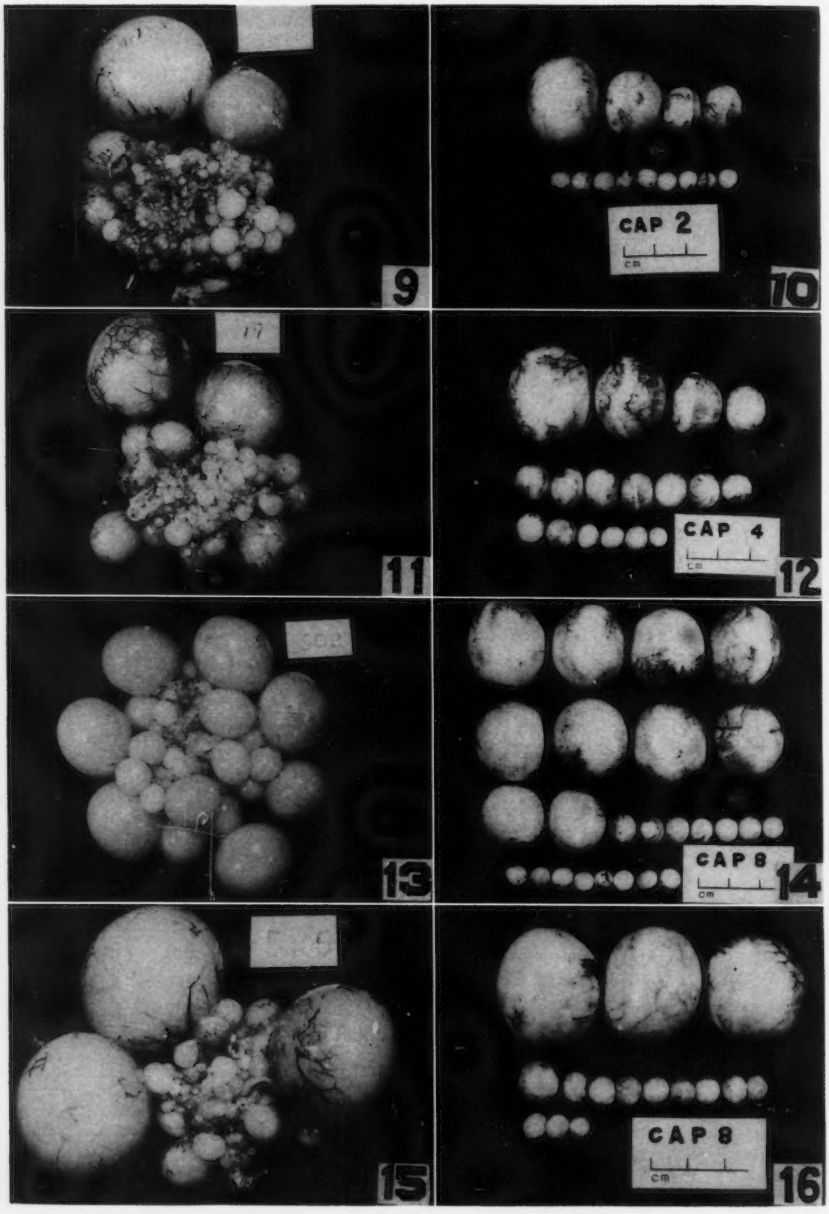


FIG. 1. Ovary of normal laying hen. Figs. 3, 5, 7. Ovaries of hypophysectomized hens treated with FSH for 2, 4 or 8 days. Figs. 2, 4, 6, 8. Hierarchies of follicles from ovaries shown in Figs. 1, 3, 5 and 7.



FIGS. 9, 11, 13 and 15. Ovaries of hypophysectomized hens treated with CAP for 2, 4, 8 and 8 days. Figs. 10, 12, 14 and 16. Hierarchies of follicles from ovaries shown in Figs. 9, 11, 13 and 15.

seen in normal laying hens (compare Fig. 1 and Fig. 7). The major portion of the total yolk mass in 6 other hens was distributed among 6 to 8 large follicles of approximately the same weight (Fig. 14). The maximum size attained by these follicles was *ca.* 8 gm. In the remaining birds, there was an increase in the number of follicles weighing 3 to 6 gm. and an apparently normal distribution of follicles in the other size classes.

Because of the considerable differences in follicular gradation among CAP treated birds, it was thought advisable to check some of the hens for the completeness of hypophysectomy. A microscopic examination of serial sections of the region of the sella turcica in 6 hens killed on Day 8 revealed no trace of anterior pituitary remnants. The reason for the observed variations between hens remains unknown.

Ovulation following OIH injection

The number of ovulations obtained from the sequence of eight daily injections of OIH did not differ significantly with treatment. The pooled results show (Table 4) that 97% of the hens ovulated in response to the first injection, and 81% ovulated as a result of the second injection. The sharp reduction in the number of hens ovulating after the second day (Table 4) was due to the relative lack of follicles of ovulable size.

The number of hens ovulating on two or more consecutive days is given in Table 5. No statistically significant difference was found between treat-

TABLE 4. INCIDENCE OF OVULATION FOLLOWING DAILY INTRAVENOUS INJECTION OF OIH INTO HYPOPHYSECTOMIZED GONADOTROPIN TREATED HENS

Treatment	Days after hypophysectomy							
	1	2	3	4	5	6	7	8
FSH	31/33*	28/33	10/22	3/17	2/9	1/9	0/4	0/4
PMS	31/31	25/31	10/23	4/21	1/11	1/9	0/5	0/4
CAP	36/37	29/37	12/29	4/26	4/18	5/18	2/10	2/10
% of hens ovulating	97.0	81.1	43.2	17.1	18.4	19.5	10.5	11.1

* Values are expressed as the number of hens ovulating out of the number surviving to the end of the day specified.

TABLE 5. NUMBER OF HENS OVULATING ON CONSECUTIVE DAYS

Number of consecutive ovulations	Treatment					
	FSH		PMS		CAP	
	No. of hens	%	No. of hens	%	No. of hens	%
2	18	54.6	16	51.6	18	48.6
3	7	31.4	9	39.1	7	24.1
4	1	5.9	1	4.8	3	11.5
5	1	11.1	0	0.0	1	5.5

ments in the number of sequences obtained, or in the number of ovulation per sequence. The pooled results show that two or more consecutive ovulations occurred in 77% of the treated hens.

A number of hens exhibited variable responses to successive OIH injections (Table 6). Several factors seem to account for the observed lapse of 1 to 5 days between ovulations. In hens 390 and 396, a double ovulation preceded a lapse of a single day, suggesting that the supernumerary follicle was prematurely ruptured and the next OIH injection failed because of the absence of an ovulable follicle. The missed days of ovulation in hens 448

TABLE 6. PATTERNS OF OVULATION OF CERTAIN HENS LISTED IN TABLE 4

Hen #	Treatment	Sequence of OIH injections							
		I1	I2	I3	I4	I5	I6	I7	I8
390	PMS	×	××	—	×				
392	PMS	×	—	×					
395	PMS	×	×	—	×	×	×		
396	PMS	××	—	×					
477	PMS	×	×	—	×				
448	FSH	—	×						
434	FSH	—	—	×	×				
482	FSH	×	××						
483	FSH	×	×	×	—	—	×		
498	CAP	×	—	—	—	—	×		
501	CAP	—	—	×					
513	CAP	×	×	—	—	—	—	—	×
514	CAP	×	—	—	—	×			
522	CAP	×	××						
524	CAP	×	×	×	—	—	×		
525	CAP	×	—	—	—	×	—	××	
527	CAP	×	×	×	—	—	×		
529	CAP	×	×	—	—	—	×	—	×
532	CAP	×	×	—	—	—	—	×	
533	CAP	×	×	—	—	×			

Ovulation following an injection of OIH is indicated by the sign ×. The dash signifies a missed ovulation.

and 501 were apparently due to atresia of the mature follicles. Examination of the ovaries indicated that the period of lapse in some hens was due to the absence of follicles of ovulable size. It is possible but improbable that an occasional ovulation was not detected because the ovum was not picked up by the infundibulum and was consequently absorbed in the body cavity (9).

Ova obtained by the first 4 OIH injections (I1 through I4) weighed 0.6 to 0.7 gm. less than did the ova ovulated prior to hypophysectomy (Table 7), while the weights of yolks in similar sequence positions in intact control hens remained unchanged in comparison to C2 ova.

Linear regression of ovum weight (including the I4 ova) on sequence position showed the regression coefficients of the treated groups to be significantly greater ($P < 0.01$) than that of the controls, but the differences

TABLE 7. MEAN WEIGHTS OF OVA (GM.) PRODUCED BY HYPOPHYSECTOMIZED GONADOTROPIN-TREATED HENS IN RESPONSE TO A DAILY TIMED SEQUENCE OF OIH INJECTIONS

Treatment	Normal ovulation		Induced ovulation				Reg. coeff. (b)
	C1	C2	I1	I2	I3	I4	
None**	16.6	16.3	16.4	16.2	16.2	16.3	+0.035*
FSH	16.5	16.3	15.7	14.7	13.0	11.9	+0.758
CAP	15.9	15.8	15.2	14.5	13.3	12.9	+0.423
PMS	17.2	17.0	16.3	15.3	14.4	14.7	+0.566

* $P < 0.01$ with all others.
** Intact, untreated control hens; weights of ova I1 through I4 are from normally ovulated C3 through C6 follicles.

between the three hormone treatments were not significant. In FSH treated hens one I5 and one I6 ovum weighed 8.9 and 12.7 grams respectively, while an I6 ovum in the PMS treated birds weighed 9.8 grams. In the CAP group the I5 injection produced 2 ova with a mean weight of 14.1 grams; the I6, 4 ova weighing on the average 11.5 grams; the I7, 3 ova weighing 11.4 grams,; and the I8, 2 ova weighing 11.9 grams.

The results (Tables 6 and 7) indicate that the intramuscularly injected hormones were unable to develop follicles to maturity as rapidly as they were being ovulated. Corroborative evidence for this assumption is supplied by the measurements of the three largest remaining follicles on the ovaries of hens autopsied at varying intervals after hypophysectomy (Table 8). The mean weights of the three follicles were, without exception, much smaller than the mean weights of the same follicles in unoperated hens. The control birds were all killed at the same point in the clutch at which the treated hens were hypophysectomized, *i.e.*, at 18 hours prior to

TABLE 8. MEAN WEIGHT OF THE THREE LARGEST REMAINING FOLLICLES IN THE OVARIES OF TREATED HENS KILLED 2 TO 8 DAYS AFTER HYPOPHYSECTOMY

Treatment	Day killed	Wt. of remaining follicles (gm.)		
		R1	R2	R3
Control	0	14.1	11.3	7.8
FSH	2	*11.3	6.1	2.9
	4	6.9	4.8	2.4
	6	8.6	6.2	2.9
	8	5.3	4.1	3.5
PMS	2	10.8	6.8	4.7
	4	10.5	6.7	4.0
	6	7.3	5.5	3.6
	8	7.9	6.4	4.7
CAP	2	10.1	5.7	2.6
	4	11.0	6.3	3.1
	6	10.0	6.4	5.2
	8	8.4	7.5	7.3

* Where atresia occurred the weight of the largest follicle was estimated and placed in sequence position on the basis of the assumed weight.

estimated hour of C3 ovulation. In general, the weights of the three follicles were smallest in the hens killed at the end of the eight day experimental period.

DISCUSSION

The fact that comb and oviduct size did not change greatly over the 8-day experimental period indicates that the selected levels of mamalian or avian gonadotropins maintained the production of ovarian hormones. The responses to FSH indicate that it was contaminated with enough LH to cause secretion of androgen and estrogen. Each of the treatments was also able to sustain, or even increase, the daily quantity of yolk deposited in the follicles.

An important part of this investigation was a comparison between the effects of gonadotropic hormones of mammalian and avian origin on the rate of growth of follicles of various sizes. None of these preparations, when administered to hypophysectomized laying hens, was capable of sustaining the hierarchy of developing follicles found in the intact hen. All three gonadotropins were deficient in one important respect: they could not maintain the normal rate of growth of larger follicles present at the time of hypophysectomy. This was clearly shown by the observations that the weights of successive ova in the ovulatory sequence declined and that the termination of a sequence in most birds was due to the absence of a follicle of ovulable size.

The differential effects of administered gonadotropins in stimulating follicular growth in hypophysectomized laying hens may be summarized as follows:

1. FSH caused a marked progressive increase in the total number of follicles entering the phase of rapid growth and a significant accumulation of small follicles (0.09 to 6.0 gms). In counting the follicles in each size class at varying intervals after hypophysectomy, it is apparent that there was a general slackening of growth as a very few follicles moved into the higher size classes. If it is assumed that the follicles found in the largest size class on Day 8 represent follicles entering the phase of rapid growth on the first and second day of treatment, the maximum possible weight increase in any follicle would be *ca.* 9 grams. By comparison, follicles entering the phase of rapid growth in the intact laying hen would develop to the normally ovulable size of 15 or more grams (10) in 7 to 9 days.

2. The pattern of follicular growth established by PMS was essentially similar to that caused by FSH. PMS brought about an increase in the total number of follicles, due to an accumulation of small follicles. The proportion of follicles found in the larger size classes was approximately the same in either case, but the largest follicles were from 1 to 2 gm. heavier in the PMS treated birds.

3. CAP caused no significant increase in the total number of follicles in

the hierarchy and no accumulation of follicles in the initial stages of rapid growth. The fact that ovulations were induced by OIH injection on the seventh and eighth day after hypophysectomy demonstrates that the stimulated follicles reached maturity within the experimental period.

The striking differences between the populations of follicles resulting from the injection of avian as opposed to mammalian gonadotropins support the hypothesis (2, 4, 11) that the gonadotropins of chicken pituitaries differ qualitatively from mammalian hormones. CAP has been shown by Taber *et al.* (4) to contain TSH and ACTH, and it is possible that these hormones play a role in yolk deposition.

It should be noted that the total amounts of yolk mobilized by the different hormone preparations used do not differ significantly (as judged by total ovarian weights), but there appears to be a significant difference in the ability of the mammalian vs. the avian gonadotropins to distribute the yolk mobilized to the follicles of different sizes. The evidence at hand is inadequate to determine whether the growth of follicles which can be elicited by the mammalian hormones is limited to some maximum size. There is no easy solution to this problem. Treatment for 8 days causes the follicles to undergo degeneration. Attempts to accelerate the rate of follicular growth by increasing the daily quantity of hormone injected failed because of atresia resulting from overstimulation of the follicles.

FSH was least able to induce growth of large follicles, PMS was noticeably superior to FSH in this respect, but the best results were obtained with CAP. Reasoning by analogy to the hormone requirements for final growth of mammalian follicles, these observations suggest the possibility that LH is essential to growth of the large follicles and that an adequate rate of growth of the large follicles requires the presence of FSH and LH in the proper ratio. Admittedly, the data presented here are not adequate to support this conclusion. What seem to be needed are investigations in which purified avian gonadotropins are tested in various combinations.

It is interesting that in about 25% of the hens given CAP for 6 to 8 days the follicles were segregated into a series of graded sizes resembling the follicular hierarchy seen in intact laying hens. The fact that a near-normal hierarchy could be established and maintained in some hypophysectomized hens receiving a single daily injection of CAP lends support to the possibility that the mechanism regulating follicular gradation resides in the ovary (12, 13).

One additional aspect of the problem is the ability of a properly timed sequence of OIH injections to induce a sequence of single daily ovulations. The data show that the OIH injections were equally effective in hypophysectomized hens given avian or mammalian gonadotropins for support of follicles. The length of the sequence of ovulations in the great majority of hens was limited by the ability of FSH to develop follicles to maturity as rapidly as they were being ovulated. The most interesting observation is

the fact that the follicles were ovulated singly, and that maturation of any follicle in a sequence occurred at some interval after the injection of OIH for ovulation of its predecessor. Thus, the sequential maturation of follicles in the hypophysectomized hen was occasionally similar to that observed in the regularly laying hens. Since the sequential maturation of as many as five follicles could be induced in hypophysectomized hens given mammalian gonadotropins, it seems highly unlikely that pituitary hormones other than FSH or LH are required to bring the follicle to the ovulatory state.

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OVULABILITY OF OVARIAN FOLLICLES IN THE HYPOPHYSECTOMIZED HEN[1]

H. OPEL[2] AND A. V. NALBANDOV

Department of Animal Science, University of Illinois, Urbana

ABSTRACT

A single intravenous injection of mammalian LH administered to hypophysectomized laying hens at 3 to 15 hours after operation forced the ovulation of two ova. The percent of hens ovulating twice was greatest (87%) when LH was given six hours postoperatively. Only single ovulations were induced in birds receiving LH within 2 hours following pituitary removal. The number of hens ovulating when injected at intervals greater than 12 hours declined sharply due to the onset of visible follicular atresia. The minimum effective level of LH for a single or double ovulation was considerably less at 12 hours than at 6 hours after hypophysectomy. The implied role of an incipient atresia of the follicle wall in the normal ovulatory process is discussed.

IN THE intact laying hen an interval of 8 to 14 hours elapses between the episodic release of the ovulation-inducing hormone (OIH) (here for the sake of simplicity assumed to be the luteinizing hormone, LH) and the rupture of a single follicle, the largest one present in the ovary (1, 2). Although the mechanism involved in ovulation has been the subject of numerous investigations, the LH-initiated changes in the follicle which terminate in its rupture remain unknown.

Kraus (3), in examining the factors involved in the ovulatory mechanism in the hen, rabbit and frog, concluded that the one condition indispensable to ovulation in these species was "regressive morphological changes in the stigma region caused by hormone action." Rothchild and Fraps (4) found that in the hypophysectomized hen the reactivity of the mature follicle to exogenous gonadotropins increases with increasing time after pituitary removal and is apparently related to the onset of atresia.

The present study demonstrates that, within certain intervals following hypophysectomy, immature as well as mature follicles become increasingly sensitive to a single injection of LH with resultant multiple ovulations.

METHODS

One hundred sixty-four regularly laying White Leghorn hens were used. The birds were maintained in individual laying cages with constant 14-hour light days and were

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[2] Present address: Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

offered a commercial laying ration and water *ad libitum*. To be certain that ovulations occurring after hypophysectomy were due to injection of exogenous LH and were not due to endogenous LH released prior to operation, hypophysectomy was performed about 18 hours prior to the estimated time of ovulation of the third (C3) follicle in an ovulatory sequence. Determination of the time of ovulation from the past records of lay was in accordance with procedures described in another publication (7). To make sure that the ovary contained a mature and at least one nearly mature follicle at the time of hypophysectomy, hens laying four or more eggs per clutch were used in this study.

To test the effect of time after hypophysectomy on ovulation, a standard dose of 4.0 mg. of a purified mammalian LH (Armour, Lot R 377279) in 0.2 cc. of saline was given by single intravenous injection at one of the intervals shown in Table 1, some hens being injected at the time of hypophysectomy, others as late as 24 hours after operation. The increasing sensitivity of follicles to LH was demonstrated by comparing the minimum effective dose for single or double ovulations at 6 or 12 hours following pituitary removal.

TABLE 1. RELATIONSHIP OF INTERVAL BETWEEN HYPOPHYSECTOMY TO EFFICIENCY OF OVULATION IN RESPONSE TO A SINGLE LH INJECTION

Interval (hrs.)	No. of hens	No. of single ovulations	No. of double ovulations	Average weight of ova (gm.)			
				C1	C2	I1	I2
0	8	8	0	17.8	17.8	16.9	—
2	8	8	0	18.4	17.7	16.3	—
3	8	6	2	17.0	16.3	14.9	7.7
4	8	6	2	18.4	17.4	15.0	11.1
6	8	1	7	17.6	17.0	15.4	13.7
12	8	2	5	17.5	17.2	14.9	10.2
15	8	5	3	14.9	14.6	12.1	9.8
18	8	5	0	17.1	16.8	10.2	—
24	8	1	0	17.0	16.8	11.3	—

C1 and C2 last normal ovulations before hypophysectomy.
I1 and I2 ovulations induced after hypophysectomy.

All hens were killed at varying times after hypophysectomy but always 14 hours after the injection of LH. The freshly ovulated ova and recently ruptured follicles were counted and all unovulated follicles were examined for gross evidence of atresia. The individual weights of ova recovered from each hen at autopsy were compared to the weights of the two ova ovulated normally immediately prior to hypophysectomy.

RESULTS

Multiple ovulations following hypophysectomy

Each hen receiving LH at 0 or 2 hours after hypophysectomy ovulated only a single ovum, but an increasing number of birds injected 3 to 15 hours after operation ovulated two ova (Table 1), and one hen injected with LH 12 hours after hypophysectomy produced three ova. The frequency of multiple ovulations decreased in hens injected later than 12 hours after hypophysectomy, but so did the frequency of single ovulations. This failure of ovulation was due to the fact that follicles remaining without hormonal support for prolonged periods become visibly atretic and deformed and the follicle wall does not rupture and release the ovum.

When multiple ovulations occurred, the smallest ova (I2) weighed sig-

nificantly less than did ova of normal ovulatory size (Table 1). In hens ovulating once in response to LH injections at 18 or 24 hours the ova were also unusually small. In these birds the mature follicles had not ovulated and were atretic on autopsy.

On statistical analysis (method of least squares) of the frequency of double ovulations with increasing interval after hypophysectomy, the data were found to fit a curvilinear regression described by the equation: $Y = 8.436 - 1.0010\ t + 0.5404\ t^2$ where Y is the probit of the percent of hens giving double ovulations in relation to time (t) (Fig. 1).

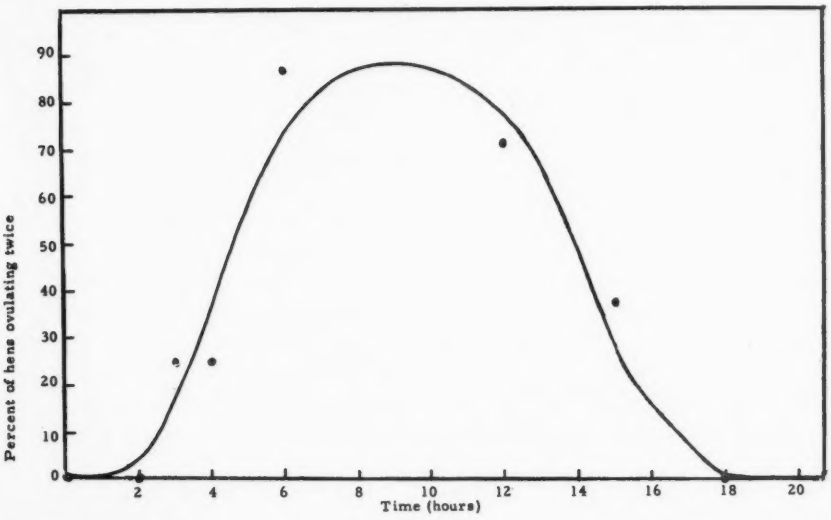

FIG. 1. Frequency of multiple ovulations in relation to time after hypophysectomy. Time scale indicates interval (in hours) between hypophysectomy and LH injection.

Minimum effective level of LH for ovulation

When LH was injected 6 hours after hypophysectomy no ovulations were induced with doses below 0.50 mg. of LH and the quantity of LH needed to force double ovulations was substantially higher (Table 2). By contrast, when LH was injected 12 hours postoperatively a dose of 0.20 mg. was sufficient to induce single ovulations, somewhat larger doses being required to cause double ovulations. Increased follicular sensitivity to LH with increasing interval from hypophysectomy to LH injection is further demonstrated by the fact that 0.25 mg. of LH, or one half the level required for single ovulations at 6 hours, was adequate to force a double ovulation in one of six hens injected at 12 hours. Mammoth doses of LH (up to 20.0 mg.) injected at 6 hours postoperatively had no effect on the number of ovulations induced.

TABLE 2. EFFECT OF TIME AFTER HYPOPHYSECTOMY ON DOSE OF LH REQUIRED TO INDUCE SINGLE OR DOUBLE OVULATIONS

Dose of LH (gms.)	6 hrs. after operation			12 hrs. after operation		
	No. of hens	No. of ovulations		No. of hens	No. of ovulations	
		Single	Double		Single	Double
0.15	—	—	—	6	0	0
0.20	—	—	—	6	2	0
0.25	6	0	0	6	2	1
0.50	6	4	0	6	3	3
1.00	6	4	2	6	2	4*
1.50	4	2	2	—	—	—
2.00	6	0	6	—	—	—
4.00	4	0	4*	—	—	—
6.00	4	0	4	—	—	—
8.00	4	0	4	—	—	—
20.00	4	1	3	—	—	—

* One hen ovulated 3 ova.

Recovery of ova from the oviduct

In approximately 50% of the hens in which multiple ovulations were induced the infundibulum picked up all of the ova ovulated. This observation is of particular interest since in experiments in which multiple ovulations are induced in normal intact laying hens only a single ovum is ever found in the oviduct (5). In the present study, when two or more eggs were found in the oviduct, the ova were, with one exception, separated in the tract by a distance of several centimeters and formed single yolked eggs (Fig. 2). This fact, plus the finding that the largest ovum was invariably the first to be engulfed by the infundibulum, suggested that the follicles did not rupture simultaneously and that the immature follicles discharged their ova by as much as four hours after ovulation of the mature follicles. However, direct observation of ovulation in four hens, given LH 6 hours after hypophysectomy and laparotomized shortly before ovulation, indicated that the rupture of the two follicles occurred either simultaneously or within a few minutes of one another.

DISCUSSION

These results demonstrate that the sensitivity of the mature follicle to exogenous LH increases with increasing time after hypophysectomy and that it appears to be related to the onset of a "physiological" follicular atresia. Of greater significance, however, is the demonstration that immature follicles, some of which are normally not destined to ovulate until three or more days later, can be regularly forced to rupture in hypophysectomized birds. The sensitivity of the obviously immature smaller follicles to LH also increases with time elapsed after hypophysectomy. Moreover, such follicles remain capable of ovulation even after the mature follicle has undergone visible atresia and has lost its ability to rupture. A more exten-

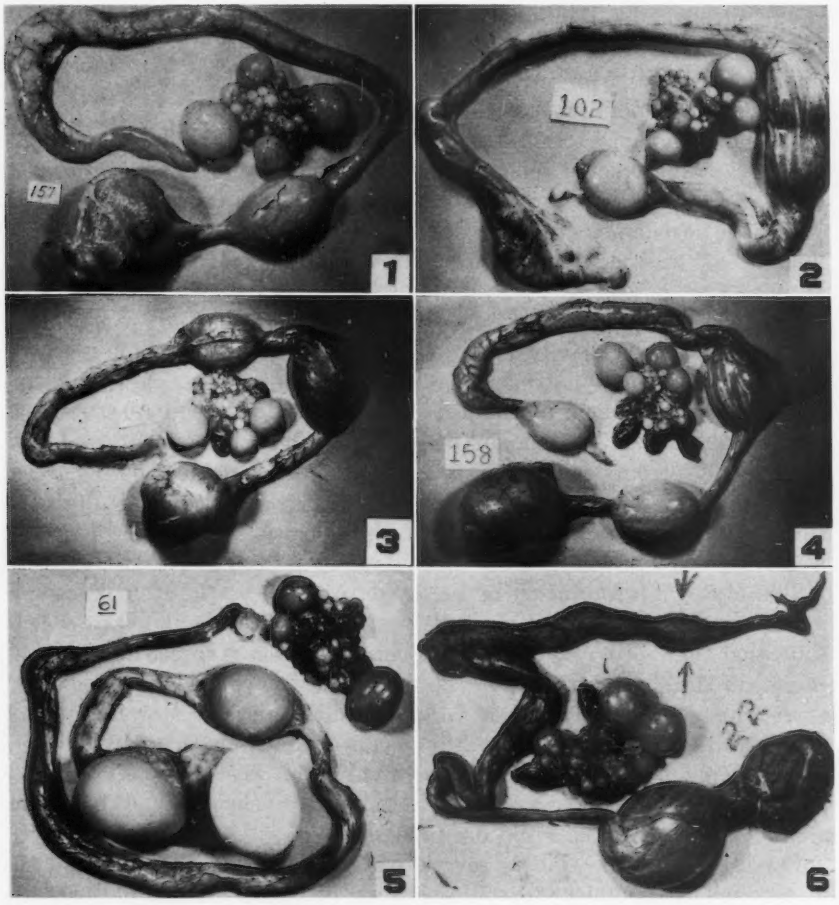

FIG. 2. Several examples of multiple LH-induced ovulations in hypophysectomized hens. Note that in some cases ova are close together in oviduct (i.e. 1 and 5), in others they are widely separated (4). Note in 6 (arrows) that a very small ovum was caused to ovulate even though larger ones are still present in the ovary.

sive investigation of this phenomenon (6) has shown that triple ovulations occur with relative frequency in hens given LH at 6 to 12 hours after hypophysectomy and that in such instances ova weighing as little as 0.8 grams can be obtained.

Attempts to induce ovulation of immature follicles in intact laying hens with a broad range of LH dosages have never been successful in this laboratory and other workers have also failed in such attempts (7). Intact laying hens given progesterone can be occasionally forced to ovulate very small follicles (7) but the endocrine mechanism involved remains unknown.

The present experiments suggest very strongly that removal of the anterior pituitary gland results in regressive follicular changes which remain undetectable grossly or microscopically, but enhance the ability of follicles to ovulate in response to exogenous LH.

In the hypophysectomized hen the gonadotropin available to all follicles is rapidly depleted following pituitary removal. Before the onset of visible atresia the follicles are, for a time, ovulable in response to exogenous LH. Since degenerative changes do not progress at the same rate or are not initiated at the same time in all follicles, only 2 or 3 follicles are ovulable at any one time.

This concept is supported by the preliminary evidence (6) which shows that follicles which have become ovulable six hours after hypophysectomy can be made non-ovulable by the administration of follicle-stimulating preparations given at the time of LH injection. However, in these experiments the largest follicle remains ovulable and all that the injection of FSH at hypophysectomy can do is to prevent multiple ovulations. Further experiments are needed to determine whether FSH-support given prior to LH injection can prevent the onset of physiological atresia (in contrast to visible atresia) of the largest follicle and render it unovulable.

The above interpretation of the ovulatory process is open to several immediate objections. First of all, there is not yet any adequate evidence to show that the vascular network of the mature follicle is sufficiently reduced to cut off the supply of gonadotrophins to the follicle cells (9). Secondly, there is no evidence to show that LH causes incipient macro- or microcsopic atresia in the follicle wall. It will be recalled, however, that Kraus (3) in investigating factors thought to be involved in the ovulatory process, concluded that regressive morphological changes in the region of the stigma are indispensable to the ovulatory process.

Finally, the observation by several workers (10, 3, and others) that the mature follicle cannot be forced to rupture by increased internal pressure applied shortly before expected ovulation, makes it unlikely that ovulation results solely from LH-induced ischemia. It may be inferred, however, that increased tension of the bands of smooth muscle surrounding the stigma are a contributing factor in follicular rupture. Although there is no proof, the stimulus to contraction of the musculature might be provided by the elaboration of some active agent, such as histamine or acetylcholine, which is released by the follicle cells undergoing necrosis.

Experiments are in progress to study the ovulatory process in birds and in mammals and to provide additional information on the problems which remain unsolved.

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INHIBITION OF RAT PITUITARY THYROTROPHIC, GONADOTROPHIC AND GROWTH HORMONE ACTIVITY WITH PITUITARY ANTISERUM[1]

T. HAYASHIDA,[2] R. RANKIN, G. McCLELLAND AND A. N. CONTOPOULOS

Department of Anatomy, School of Medicine, University of California, San Francisco, California

ABSTRACT

A potent antiserum against rat pituitary thyrotrophic, gonadotrophic, and growth hormones has been produced in rabbits by their long term immunization with rat pituitary saline homogenates. The procedures used were similar to those we have previously employed with highly purified human and bovine pituitary growth hormones. The detection of antibodies to particular hormones was based largely upon results of antihormone tests performed in hypophysectomized rats injected with the antiserum. It was observed in such animals that the antiserum could neutralize the stimulating effects of the rat pituitary homogenates upon thyroid morphology and I^{131} uptake, gonadal development, and body growth. Evidence is thus presented for the production of antibodies to anterior pituitary hormones in their crude, natural, or near natural state. In order to strengthen the validity of the specific antihormone effects of the antiserum, particular emphasis is placed upon the control of non-specific or toxic influences of the antiserum.

THE production of an antiserum to a highly purified bovine pituitary growth hormone was reported in 1958 (1, 2). This was soon followed by the successful production of potent antisera to highly purified human pituitary growth hormone (3, 4). The antihormone effect of the antiserum, or its capacity to inhibit the biological activity of the homologous growth hormone, has been demonstrated (1, 2, 4, 5) and confirmed by several workers (6, 7, 8). The specificity of this growth hormone antigen-antibody reaction was strongly suggested through results obtained by several investigators employing various immunological techniques (2, 4, 5, 6, 7, 8, 9).

In the present study, the presence or absence of antibodies to particular hormones is based largely upon results of antihormone tests. Immunization techniques similar to those used previously with highly purified growth hormone have been employed. Evidence is presented for the production of antibodies to anterior pituitary hormones, not in their purified form in which they have undergone various physico-chemical manipulations, but

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[2] Senior Research Fellow of USPHS. (SF-78).

as they exist in their crude, natural or near-natural stage. Particular attention has been given to the control of non-specific or toxic effects of the antiserum, an aspect which has not received adequate attention in most studies of a similar nature which have dealt with crude antigens (10, 11). A preliminary report of the present findings has been presented elsewhere (12).

MATERIALS AND METHODS

Animals

In the present studies young, male albino rabbits, weighing 2.6 to 2.8 kg. were employed for the production of antiserum. They were maintained on a standard laboratory feed,[3] supplemented once weekly with fresh greens.

Young, male rats of the Long-Evans strain, were hypophysectomized at 40 days of age and were employed for the antihormone tests 3 weeks and 5 months post-operatively. Immature female rats of the same strain, hypophysectomized at 26 to 28 days of age were also used, 3 weeks post-operatively. The hypophysectomized rats were kept in a constant temperature room at $75° \pm 2°$ F, and were maintained on a standard diet.[4] All rats were weighed on the first day of experiment and every other day thereafter. At autopsy the completeness of hypophysectomy was checked in all animals by examination of the sella turcica with the aid of a binocular dissecting microscope.

Preparation of antiserum

Rabbits were immunized with 60 to 80 rat anterior pituitary lobes per rabbit, administered in single doses of 5 to 10 glands in a series of injections extending over a period of 6 months to a year and a half. These pituitaries, obtained from adult male and female rats of various ages, were fresh frozen and stored at $-20°$ C until use. They were homogenized in 0.85% saline in a Teflon tissue homogenizer, adding 0.05 cc. of n-butyl alcohol in order to minimize foaming and to serve as a preservative. The amount of n-butyl alcohol was adjusted by volume to a final concentration of 1 to 2%. Equal volumes of the pituitary-saline phase and of an oily adjuvant were then emulsified to a lanolin-like consistency with the aid of a hypodermic syringe. The initial injection of the rabbits with rat pituitary homogenate was made with complete Freund's adjuvant (Difco) while in the subsequent injections, Bayol-Arlacel (4 to 1 by volume) was used as adjuvant. One-half of the initially injected dose was given subcutaneously and the other half, intraperitoneally. After this initial injection all subsequent injections were made subcutaneously. At roughly 3 month intervals, complete Freund's adjuvant was substituted for Bayol-Arlacel. The rabbits were bled by cardiac puncture approximately 16 to 18 days after the last antigen injection. The sera were obtained from the blood samples and stored at $-20°$ C until use, at which time merthiolate was added for a final concentration of 1:10,000.

The pooled antiserum prior to use was absorbed free of certain non-specific antibodies. The antiserum was absorbed with normal rat plasma for $1\frac{1}{2}$ hours (Fig. 1), centrifuged, and absorbed for another $1\frac{1}{2}$ hours with rat red blood cells and concentrated rat liver

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[3] Prepared by Simonsen Company, Gilroy, California.

[4] Hypophysectomized rats used in assays were maintained on a modified McCollum Diet I, consisting of ground whole wheat 67.5%, casein 15%, skim milk powder 7.5%, sodium chloride iodized 0.75%, calcium carbonate 1.5%, melted fat 6.75%, fish oil (Vit. A and D concentrate) 1%. KI solution added to 1 μg of iodine per gram of diet (450 mg. KI per liter, 300 cc. per 270.5 lbs of diet). In addition a dish of wet mash of this diet was offered every afternoon. They received a supplement of lettuce once weekly.

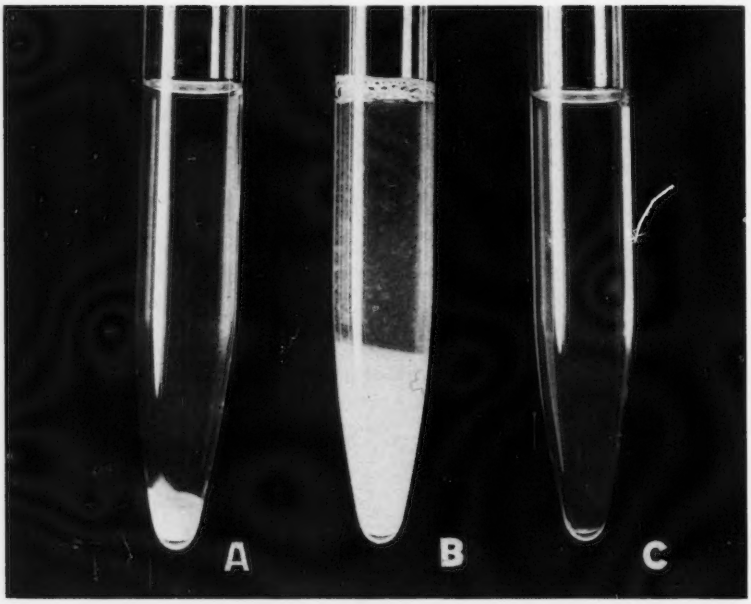


FIG. 1. Absorption of RPH antiserum with normal rat serum. Normal rat plasma (0.2 cc. of 1:10 dilution in saline) was added to each 8.0 cc. aliquot of antiserum (A and B) and normal rabbit Serum (C). Note formation of large amount of precipitate in A and B, after and before centrifugation respectively, and absence of precipitation in C.

homogenate. All absorptions were carried out in a 37° C water bath. The efficacy of this absorption procedure was tested by the double diffusion agar technique of Ouchterlony (13), in which the tests were read after 72 hours incubation at room temperature. It was found that when the unabsorbed antiserum was allowed to react with normal rat plasma, six to seven different precipitation lines appeared. After absorption, however, the antiserum did not give a single reaction line with rat plasma although 3 lines still developed when it was reacted with the crude, rat pituitary extract. It was this absorbed antiserum which was employed in all antihormone tests.

Antihormone tests

Antihormone tests were performed in hypophysectomized male or female rats, as indicated under each experiment. The rats were given daily subcutaneous injections of rat pituitary homogenate (RPH); the RPH was prepared from fresh frozen anterior pituitaries obtained from adult male rats between 5 months and 1 year of age. The daily dose of RPH prepared as described above was equivalent to $\frac{1}{4}$ anterior pituitary per rat, a dose producing significant stimulation of various pituitary target organs. In addition, some of the rats were injected intraperitoneally with RPH antiserum (A/S), in order to determine whether or not any of the target organ responses to RPH could be inhibited. Other rats received intraperitoneally, normal rabbit serum (NRS) in addition to RPH. All injections were continued daily for 4 to 8 days according to the particular experiment. The animals were sacrificed 24 hours after the last injection. Eighteen hours before autopsy they were injected intraperitoneally with 3 μc of I^{131}. At autopsy the thyroids

were dissected and placed in counting vials containing Bouin's fluid for fixation. They were counted for radioactivity in a scintillation counter (14). The results are presented as percent I^{131} uptake of the injected dose. The thyroids were later sectioned and stained with hematoxylin and eosin for histological examination. The testes were removed, weighed, fixed in Bouin's, embedded in paraffin, sectioned at 6 μ and stained with iron hematoxylin and aniline blue. The tibias were removed, split in halves and fixed in 10% neutral formol for determination of the width of the proximal epiphyseal cartilage plate by the method of Greenspan *et al.* (15).

EXPERIMENTAL AND RESULTS

Experiment I

Male rats hypophysectomized at 40 days were employed 5 months post-operatively. They were uniformly distributed on the basis of body weight into 3 groups of 5 animals each. The first group consisted of non-injected hypophysectomized controls. The rats of the second and third groups received daily, for eight days, subcutaneous injections of rat pituitary homogenate equivalent to $\frac{1}{4}$ adult male anterior pituitary. In addition, the animals of the second group received daily intraperitoneal injections of 1 cc. of normal rabbit serum (NRS), while those of the third group were injected by the same route with 1 cc. of the antiserum (A/S) instead of NRS. All animals were sacrificed on the 9th day for study of their target organs.

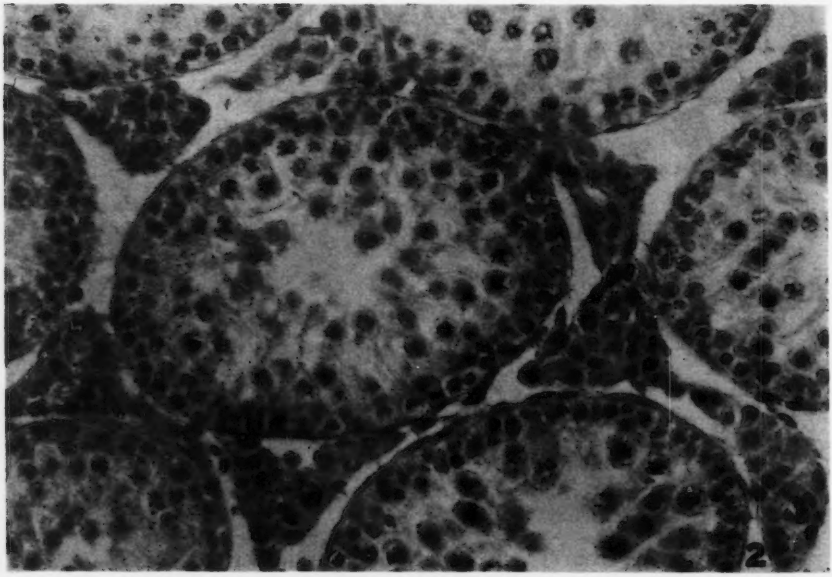
It may be noted from Table 1 that the daily injections of RPH+NRS resulted in a highly significant increase in testicular weight to a value more than double that of the testes from non-injected controls. The injection of A/S into the rats in addition to RPH, completely inhibited this testicular response. Histologically it was observed that the RPH+NRS treatment resulted in a very marked stimulation of the seminiferous tubular epithelium (Fig. 2). The tubules were much thicker than those in the hypophysectomized controls and exhibited numerous mitoses. The interstitial cells of Leydig were relatively large with abundant cytoplasm, presenting a picture of good stimulation. In the RPH+A/S injected animals, the tubules were as small as in the hypophysectomized controls, mitoses within them were considerably fewer in number and the interstitial cells appeared just as deficient as those in the hypophysectomized control animals (Fig. 3).

TABLE 1. EFFECT OF RAT PITUITARY HOMOGENATE RABBIT ANTISERUM (A/S) OR NORMAL RABBIT SERUM (NRS) ON THE RESPONSE OF PITUITARY TARGET ORGANS, TO ADMINISTRATION OF RAT PITUITARY HOMOGENATE (RPH)

Recipients*	No. of rats	Testes	Thyroid I^{131} uptake	Body weight increase	Tibial epiphyseal plate width
			%	gm.	micra (μ)
1. Uninjected	6	254 ± 34**	0.33 ± .06	—	106 ± 4
2. RHP+NRS	5	571 ± 39	5.10 ± .68	8.8	249 ± 33
3. RPH+A/S	5	271 ± 40	0.34 ± .08	1.2	98 ± 10

* Animals were male rats, hypophysectomized at 40 days of age and employed 5 months post-operatively.

** Standard error of the mean.



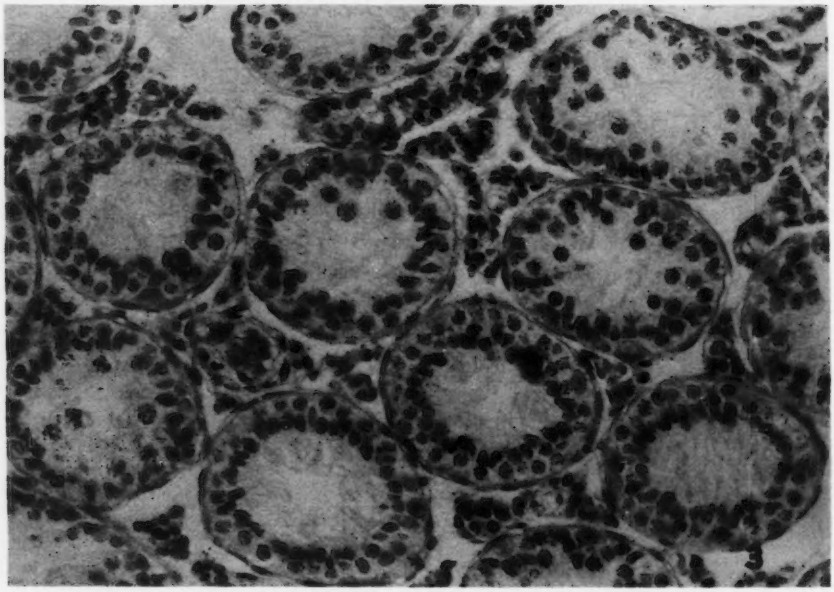


FIG. 2. Effect of RPH on seminiferous tubules and interstitial cells of hypophysectomized rat testes. ×240.

FIG. 3. Inhibition of RPH stimulation of hypophysectomized rat testes by the simultaneous administration of RPH antiserum. Histological picture is similar to that of uninjected hypophysectomized controls. ×240.

There was a very strong stimulation of the thyroid gland as judged by its I^{131} uptake in animals injected with RPH+NRS. The I^{131} uptake increased markedly from the control value of 0.33% to 5.10%. Again, the simultaneous treatment with A/S completely suppressed the uptake to a level of .34% which was not different from that of hypophysectomized controls. Histological examination of the thyroids also gave evidence for suppression of the thyroid function (Figs. 4 and 5). The thyroid glands of animals injected with RPH and NRS (Fig. 4) showed increased height of the follicular epithelium, larger and lighter staining nuclei, when compared to that of the hypophysectomized control animals. The stimulated thyroids of these animals appeared quite similar to those found in normal rats of the same age. In contrast, the thyroid glands of animals which received A/S in addition to the RPH (Fig. 5) showed a very flattened follicular epithelium with dark staining nuclei, a picture representative of extreme hypofunction. The thyroid follicles in the animals from the latter group appeared indistinguishable from those of the uninjected hypophysectomized control animals.

A very marked stimulation of the tibial epiphyseal cartilage plate occured in the RPH+NRS group; the average plate width was 249 μ as compared to 106 μ for the controls. Once more, the antiserum was capable of completely neutralizing this growth promoting effect of the RPH. All of the stimulating effects observed in the target organ responses in the RPH+NRS treated animals, as well as the inhibition of these responses with the antiserum, were of a high order of significance ($P < .001$, per "p" values of Fisher).

Thus the results of this experiment have shown that the rabbit antiserum to the whole rat pituitary homogenate could neutralize the biological activity of injected rat pituitary hormones, namely, gonadotrophic, thyrotrophic and growth hormones.

Experiment II

The second experiment was performed to test for the possible toxicity of rabbit antiserum in comparison to normal rabbit serum. Male rats hypophysectomized at 38 to 40 days of age, were employed three weeks post-operatively. The first group of rats received NRS only, while the second group was injected with A/S only. The daily dose of NRS or A/S was 0.6 cc. per rat for the first 5 days and 1.2 cc. for the last 3 days. The animals of the 3rd group received RPH and NRS while those of the 4th group were injected with RPH and A/S. The rats of the 5th and 6th groups were likewise injected with RPH but the A/S to RPH was substituted with rabbit A/S to bovine or human pituitary growth hormone.

It may be seen from Table 2 that neither the body weight change nor the tibial cartilage width showed any significant difference between the groups injected with NRS or A/S. Both responses were within the normal range of variation of uninjected controls and it was clearly demonstrated that while the A/S was capable of inhibiting the growth promoting factor(s) present in

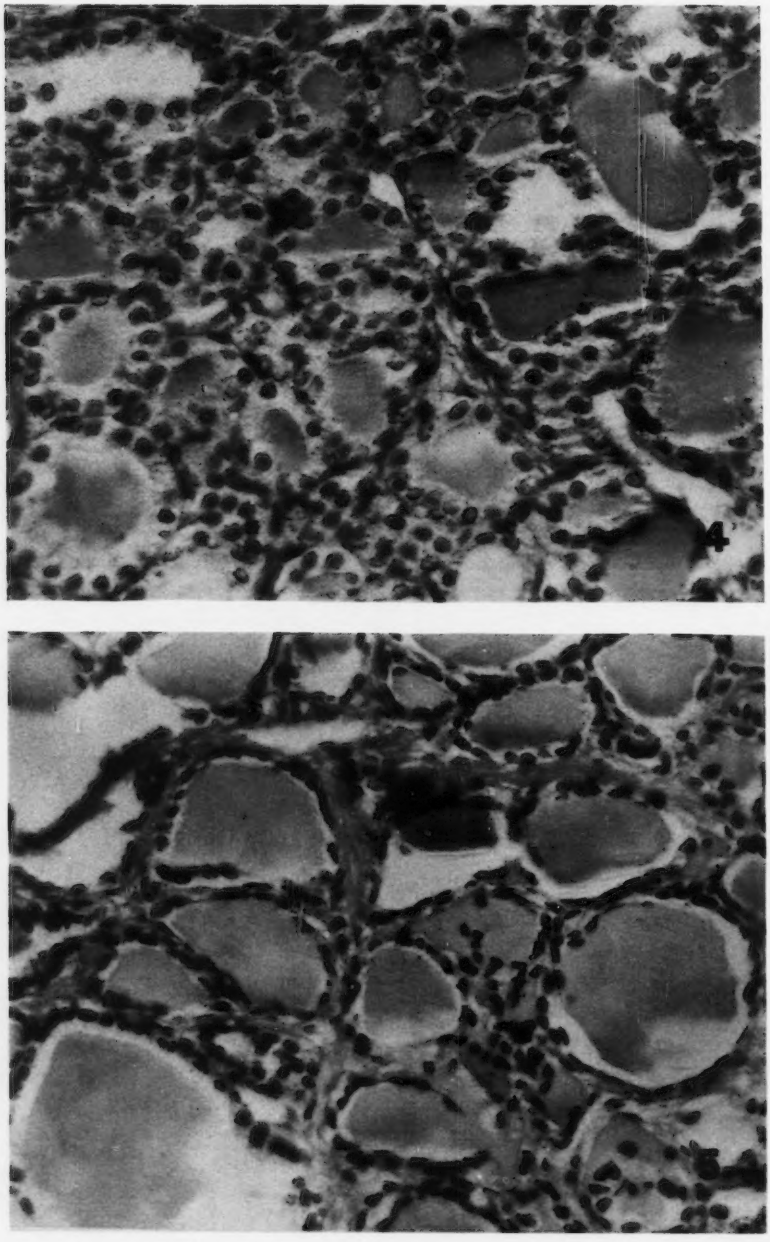


FIG. 4. Effect of RPH on the thyroid gland of hypophysectomized rat. ×300.

FIG. 5. Inhibition of RPH stimulation of hypophysectomized rat thyroid by the simultaneous administration of RPH antiserum. Histological picture is similar to that of uninjected hypophysectomized controls. ×300.

TABLE 2. ANTISERUM TOXICITY TEST AND SPECIES OF SPECIFICITY OF ANTISERUM*

Group	Body weight increase	Tibial epiphyseal plate width
	gm.	micra (μ)
1. — NRS**	5	151 ± 7***
2. — RPH A/S**	5	148 ± 10
3. RPH+NRS	28	296 ± 17
4. RPH+RPH A/S	17	218 ± 13
5. RPH+BGH A/S****	27	287 ± 9
6. RPH+HGH A/S****	30	284 ± 12

* All bioassay rats were male, hypophysectomized at 38–40 days of age and employed 3 weeks post-operatively; 5 rats in each group.

** Serum dosage, 0.6 cc./day for the 1st 5 days, 1.2 cc/day for the last 3 days. Injections of NRS or A/S alone, at the dosage employed, have not produced any significant changes in any target organ response in similar hypophysectomized rats.

*** Standard error of the mean.

**** BGH and HGH A/S = Bovine and human pituitary growth hormone antiserum, respectively.

RPH, it did not show any significant inhibitory or toxic effect when injected alone into the hypophysectomized control animals (Group 2). The rats injected with RPH+NRS (Group 3) showed a very marked body weight increase of 28 grams in 8 days (average gain of 3.5 gm. per day), and a very strong tibial cartilage stimulation to 296 μ. When A/S was injected together with the RPH (Group 4) there was a significant, although incomplete inhibition of body weight gain, and the tibial cartilage stimulation was held to a 44% increase over the NRS injected animals. It appears that the amount of A/S employed was not adequate to completely inhibit the growth promoting and tibial epiphyseal plate stimulating influence of the dosage of RPH administered, nevertheless, the inhibitory effect of the A/S was still very significant.

The animals injected with bovine or human growth hormone A/S in combination with RPH showed no inhibition of body weight gain or tibial cartilage stimulation. In other words, the antisera against bovine and human pituitary growth hormone, were not able to neutralize the rat growth hormone present in the RPH although the amount of antisera administered here (0.6 to 1.2 cc.) should have been sufficient to neutralize from 60 to 120 micrograms per day of highly purified bovine growth hormone (2).

Experiment III

Preliminary agar diffusion tests (Ouchterlony) with antiserum to human growth hormone (HGH) could not detect a substance (growth hormone) in RPH which was immunologically similar to HGH, and conversely, similar tests with RPH A/S did not reveal any visible cross reactions with HGH (16). Since these results as well as those obtained in the above experiment (Table 2, Group 6) had suggested that HGH and rat growth hormone are different in antigenic structure, it was felt that the administration of HGH

together with RPH A/S might constitute a critical test for the non-specific toxicity of the RPH A/S. It was thus assumed in this experiment, that RPH A/S would probably not inhibit HGH activity, if the antigenic structure of HGH were indeed different from that of rat growth hormone; and it was further assumed that should RPH A/S inhibit HGH activity, the result may be due to a non-specific, toxic effect of this antiserum.

The antihormone studies were performed in immature, female rats hypophysectomized at 26–28 days of age and employed 3 weeks postoperatively. One group of rats served as hypophysectomized controls (Table 3), while a second group received RPH and NRS. The third group likewise received RPH and in addition were injected with RPH A/S instead of NRS. In order to further test for the toxicity of this antiserum, the last 2 groups were injected with highly purified human growth hormone (HGH) and either NRS or RPH A/S.

TABLE 3. FAILURE OF RAT PITUITARY HOMOGENATE ANTISERUM TO INFLUENCE THE EFFECT OF HUMAN GROWTH HORMONE ON THE EPIPHYSEAL TIBIAL PLATE WIDTH*

Group	No. of rats	Tibial epiphyseal plate width
		micro (μ)
1. Controls	4	142 ± 5**
2. RPH+NRS	4	248 ± 4
3. RPH+RPH A/S	5	167 ± 4
4. HGH+NRS	4	203 ± 22
5. HGH***+RPH A/S	4	213 ± 12

* All bioassay rats were female, hypophysectomized at 26–28 days of age and employed 3 weeks post-operatively.

** Standard error of the mean.

*** HGH = human growth hormone (Li), 7.5 μg/day. All injections were given daily for 4 days; RPH and HGH were injected subcutaneously, and serum, intraperitoneally.

The results of this experiment showed that the administration of RPH plus NRS produced a highly significant increase in tibial cartilage width up to 246 μ as compared to 141 μ for the hypophysectomized controls. Most of this stimulatory effect due to RPH was again neutralized by the injection of RPH A/S (Group 3). The animals which received HGH and NRS showed significant tibial plate stimulation to 202 μ. The most significant observation was that in the last group, rats injected with the same dosage of HGH had a similar epiphyseal tibial response (212 μ) in spite of simultaneous injections of RPH A/S. These findings strongly suggested that the inhibition of the growth promoting effect of RPH with RPH A/S is not a non-specific toxic effect, but rather, a specific, immunological phenomenon.

DISCUSSION

The inhibition of biological activity of hormones in their crude state with antiserum to the crude preparation is certainly not new (10). However, it is believed that the studies presented here clearly demonstrate inhibition of the effects of pituitary trophic hormones in their crude, natural or near natural state, through use of antisera which have been well absorbed to remove

various non-specific antibodies. The antisera were absorbed with such substances as serum proteins, red and white blood cells and concentrated liver homogenate, all obtained from the rat source, the species homologous to the hormones being neutralized. Particular emphasis was given to the control of other possible non-specific or toxic effects of the antiserum.

It is felt that one of the most convincing demonstrations of the non-toxicity of the antiserum, was the finding in hypophysectomized rats that although RPH antiserum could inhibit the activity of rat growth hormone present in the RPH, it could not inhibit the activity of human growth hormone. If the inhibition of rat growth hormone activity with the RPH antiserum were due to a non-specific, toxic effect of the antiserum, then presumably the human growth hormone should likewise have been neutralized. However, this was not the case. The inhibitory effects of antiserum appear to have been induced by specific, immunological reactions rather than by non-specific or toxic influences.

We have previously reported an attempt to neutralize the endogenous pituitary growth hormone in normal young rats (5), by the injection of rabbit antiserum to highly purified bovine growth hormone. The results were negative in spite of daily administration of moderately large amounts of antiserum. However, in the normal animal, one has no control over the compensatory hypersecretion of pituitary hormone(s) by the intact hypophysis. It is quite possible that the amount of production and secretion may equal or even more than compensate for the amount of hormone(s) being neutralized in the peripheral circulation by the injected antibodies, in which case the antihormone effect of the antiserum, may be masked and therefore remain undetected. These negative results might also be explainable on the basis of the results obtained in the present investigation, in which antiserum to bovine growth hormone could not show any significant neutralization of the rat pituitary growth hormone activity. Precipitin tests in agar have given further evidence that although human and bovine growth hormone antisera can give a visible reaction with the homologous growth hormone, they could not do so with the rat growth hormone present in rat pituitary homogenate, even though the homogenate was employed over a wide range of concentrations. In fact, it was observed in the present studies that rat pituitary growth hormone purified by Dr. Harold Papkoff, and found by him to be more active in the rat than any other growth hormone ever tested in that species (17), did not give a visible reaction with antiserum to human or bovine growth hormone in the agar diffusion test (Ouchterlony). The foregoing results have further emphasized the rather high degree of species specificity that exists with respect to interaction of growth hormone with the growth hormone antibody (4, 5).

Although the present investigation has demonstrated the existence in the antiserum, of antibodies to pituitary thyrotrophic, gonadotrophic and growth hormones on the basis of antihormone tests, there was no definite

evidence of the presence or absence of antibodies to adrenocorticotrophic hormone. The presence or absence of antibodies to lactogenic hormone was not determined.

Acknowledgments

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THE HISTOCHEMICAL DEMONSTRATION OF THE SITES OF LUTEOTROPIC HORMONE IN THE RAT PITUITARY GLAND[1]

RUSSELL J. BARRNETT,[2] WILLARD D. ROTH AND JOHN SALZER[3]

Department of Anatomy, Harvard Medical School, Boston, Massachusetts

ABSTRACT

This study describes the development and tests the usefulness of a histochemical method for the demonstration of the sites of Luteotropic hormone (LTH) in rat pituitary glands. When these glands, taken from normal animals, are immersed in 0.5% trichloracetic (TCA) acid, only LTH is precipitated; the remainder of the anterior pituitary hormones are dissolved and extracted as proven by appropriate bio-assays and *in vitro* tests. When such extracted glands, containing a normal amount of only LTH, are fixed in histological fixatives, sectioned and stained by histochemical methods that specifically reveal the sites of either sulfhydryls and disulfides or carboxyl groups, only the granular cytoplasm of some of the acidophils are reactive. Basophils, chromophobes and the remainder of the acidophils are unreactive. Reduction of the concentration of TCA to 0.1%, which extracted LTH in addition to the other hormones, eliminated the histochemical reaction in all the parenchymal cells. In addition, the number of cells that reacted histochemically in the 0.5% TCA extracted glands of pregnant and lactating rats varied directly with the amount of hormone present.

THIS paper is concerned with the histochemical demonstration of luteotropic hormone (LTH) in the rat pituitary gland. The principles used for this demonstration are those previously established for the demonstration of the sites of the glycoprotein hormones (1); i.e., the combined use of differential protein solubility, histochemical staining and bio-assay. In the present experiments a reagent (0.5% trichloracetic acid) was found that was solvent for all the pituitary hormones except LTH, as proven by appropriate bio-assays. When the extracted glands were fixed in a histological fixative and stained with histochemical methods, only the granular cytoplasm of some of the acidophils was reactive. In this manner the LTH content of pituitary glands of normal adult male and female rats, as well as

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[2] Present address: Department of Anatomy, Yale University School of Medicine, New Haven, Connecticut.

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those of pregnant and lactating rats, were investigated histochemically. These results were reported briefly in previous communications (2, 3).

MATERIALS AND METHODS

In vitro experiments

In order to establish the validity of the methods used, the solubility characteristics of isolated and purified pituitary hormones[4] were studied by *in vitro* experiments as described previously (1). Although a large number of reagents, suggested by others (4–10) who studied the chemical and physical characteristics of LTH, were tested on aqueous solutions of the pituitary hormones (1 mg./ml.), only the tests involving trichloracetic acid (TCA) are pertinent to the present report. It was found on titrating solutions containing pituitary hormones with known concentrations of TCA that addition of small amounts of the titrating agent caused a white flocculent precipitate to develop only in solutions of LTH. When a final concentration between 0.25% and 0.5% TCA was reached, LTH appeared to be completely precipitated since no further precipitation could be developed from the supernatant fluid by heat or by addition of absolute acetone, saturated solutions of picric acid or mercuric chloride. At these concentrations of TCA, no precipitates developed in the solutions containing the other pituitary hormones. At final concentrations between 2.0% and 4.0% TCA, Adrenocorticotropin (ACTH), Luteinizing hormone (LH) and Growth hormone (STH) were precipitated from solution but Follicle-stimulating hormone (FSH) and Thyrotropin (TSH) remained in solution even when the concentration of TCA was raised to about 10%.

In addition, it was established that LTH was precipitated from solution by absolute acetone and by two histological fixatives, sublimate-formol and a modified Susa fluid. As indicated in the previous experiments on glycoprotein hormones (1), acetone was used to precipitate the hormone because biological activity was retained and it could be bio-assayed; whereas, LTH precipitated by the histological reagents was biologically inactive. Moreover, LTH was precipitated irreversibly by the histological fixatives for the histological conditions used to prepare sections (fixation, dehydration, embedding, and rehydration), and the histochemical methods of staining. Indeed, in model experiments it was shown that fixed LTH, carried as a precipitate, could be stained with several histochemical methods that demonstrate groups in protein (disulfide and carboxyl). Acetone could not be used for histological purposes since the acetone-precipitated LTH was soluble in the dilute organic solvents used in the histological procedures of dehydration and rehydration.

Treatment of donor pituitaries for histology and for bio-assay

The pituitary glands of over 600 donor rats were used in the preliminary and definitive experiments. The present report is based on a study of 360 of these glands obtained from normal, young, adult, male and female rats of the Hisaw strain, 175 to 250 gm. in weight, and from 76 female rats at various stages of pregnancy and lactation.

Pituitaries fixed in sublimate-formol or a modified Susa's fluid and in absolute acetone for histochemistry and bio-assay respectively, were considered the controls which contained all the hormones. Glands prepared using these procedures were considered comparable so that the results of bio-assay and histology could be equated. This conclusion was based upon the results of the *in vitro* experiments and the fact that the staining results obtained from glands fixed in acetone and refixed in sublimate-formol were essentially identical with those fixed in sublimate-formol alone.

[4] The authors are indebted to Dr. S. S. Steelman of the Armour Laboratories, Chicago, or providing samples of isolated and purified pituitary hormones.

In addition to the control fixatives referred to above, 22 solvents were tested. Although the results with TCA (at concentrations of 0.05% to 14.0%) were the most useful in the present investigations, the results obtained with several other solvents shall be mentioned in passing. The pituitary glands were rapidly removed from decapitated rats and placed in the solvent for 24 hours. These were refixed for 24 hours in two changes of absolute acetone for bio-assay or for 6 to 16 hours in one of the histological fixatives for histochemistry. The glands for bio-assay were air-dried and stored in a vacuum desiccator containing a drying agent. Two days before the assays were begun, the pituitaries were weighed, pooled, ground with a glass homogenizer, suspended in physiological saline and frozen. These suspensions contained one acetone-dried gland (av. 1.2 mg.) per 0.5 ml. The solutions were thawed for use and were kept thereafter at 3° C.

Assay materials and methods

The assays for LTH, performed on common pigeons 300 ± 40 gm. in weight, were derived from the local crop sac methods of Lyons and Page (11) and Reece and Turner (12). The former method is very sensitive (1/10,000 of a microunit) and the latter is useful for the assay of pituitaries of small laboratory animals. The pigeons were kept in individual cages and fed pigeon feed and water *ad libitum*. The feathers over their crop sacs were plucked 1 or 2 days before the assay was begun. These birds received injections of 0.1 ml. pituitary suspensions intradermally into feather follicles for four consecutive days; the site of injections being marked with India ink so that the same ones were always used. With some of the birds, injections of suspension of extracted pituitary glands were made on the extreme right over the crop; and on the extreme left, the injections consisted of suspension of unextracted acetone-dried glands, of the saline diluent or of solutions containing 4 or 16 μg of Prolactin (Armour). In the other birds, single injections were made at the front of the crop. On the fifth day the birds were killed by decapitation, crops dissected out, opened and transilluminated. The proliferative reaction underlying the site of injection was read and given a value from zero to 4. It should be noted that although the assay used was not as quantitative as other bio-assays, we nevertheless found it appropriate for several reasons. The assay is simple and the degree of proliferation that was sought was either zero (extraction of the hormone) or maximal (hormone not extracted). The methods that resulted in partial extraction of LTH or partial precipitation of the other hormones were discarded after preliminary experiments.

The remaining assays were performed on hypophysectomized Sprague-Dawley rats. Those used for the determination of the glycoproteins, FSH, LH and TSH, have been described elsewhere in the paper dealing with the localization of these hormones (1).

Additional assays were performed for Somatotropin (STH) and Adrenocorticotropin (ACTH) according to the tibia growth test of Evans, *et al.* (13) and the "Munsen modification" of the Sayers adrenal ascorbic acid response (14), respectively. These latter two assays were used to show that the methods for precipitating LTH were solvent for STH and ACTH.

Histochemical methods for donor pituitaries

Pituitaries of the donor rats used for histological study were treated as follows: controls were immersed directly in either sublimate-formol or a modified Susa's fluid. The experimental glands were placed in a solvent for 24 hours and then refixed in the above solutions. The glands were dehydrated, embedded in paraffin and strip-serial, 3 μ, horizontal sections were cut on two or three different levels. Single sections or small strips were mounted on numbered slides, deparaffinized and treated with Lugol's solution and thiosulfate to remove mercuric crystals. The sections were stained with specific methods for sulfhydryl and disulfide (SH and S-S) groups (15), α-acylamido carboxyl groups (16), or with acid dyes; the latter being used for historical reasons.

RESULTS

The results of the bio-assays of unextracted and extracted pituitaries of normal female rats are summarized in Tables 1–3.

LTH assays. When the unextracted pituitaries of normal, estrous, adult, female rats were assayed by a local crop method, the response indicated an LTH content between that given by 4 and 16 μg of an isolated and purified Prolactin preparation (Armour). Pituitaries from normal male rats appeared to contain less than 4 μg. Extraction of pituitary glands in TCA (concentrations of 0.25% to 2.5%) resulted in little or no loss in LTH potency in comparison to the acetone controls. However, when concentration of TCA in the extracting media was reduced to 0.1% or 0.05%, the pituitary glands showed a marked loss in hormone content (Table 1). In fact, 80% of the animals injected with pituitary glands extracted in these

TABLE 1. BIO-ASSAY OF UNEXTRACTED AND EXTRACTED RAT GLANDS FROM NORMAL FEMALE RATS INDICATING LTH CONTENT

Pituitaries	No. of pigeons	Average response	Range
Prolactin controls 16 μg	19	3.7	3–4
Prolactin controls 4 μg	16	2.2	1–4
Acetone control	30	3.0	2–4
0.05% TCA	8	0.1	0–1
0.1% TCA	12	0.4	0–1
0.25% TCA	12	2.4	2–4
0.5% TCA	10	2.6	2–4
2.5% TCA	11	3.0	2–4
Saline controls	8	0	—

latter media showed no response, indicating successful extraction of LTH. Although not included in Table 1, the results of several other solvents should be noted. Glacial acetic acid was as effective as 0.1% TCA in extracting LTH and distilled water was as effective as 0.25% TCA in precipitating the hormone. These reagents were not used in the final experiments since the morphology of the extracted glands was not acceptable. Ethanol (70%) as well as acetone (75%) extracted most of the LTH but these were not used since they both precipitated STH. The remainder of the reagents tested succeeding in only precipitating part of the LTH as determined by bio-assay and/or precipitating some of the STH and/or ACTH and therefore were discarded.

STH and ACTH assays. These assays, performed on extracted and unextracted pituitaries, indicated that both hormones are completely extracted by a concentration of TCA (0.5%) that precipitates the LTH (Table 2). Higher concentrations of TCA precipitate first ACTH and then STH. Although not indicated in Table 2, ACTH was solvent in both 75% acetone and 70% ethanol.

Glycoprotein Hormone Assays. Localization of these hormones has been dealt with in a previous paper (1) indicating that their sites are completely

TABLE 2. BIO-ASSAY OF EXTRACTED AND UNEXTRACTED PITUITARY GLANDS OF RATS INDICATING STH AND ACTH CONTENT

Treatment	Number of rats	Mean epiphyseal diameter in μ	S^2†	Microgram pituitary tissue injected/100 gm. body weight	Number of rats	Decrease in adrenal ascorbic acid; micrograms/mg. adrenal	% ACTH remaining
Hypox controls*	7	29.80	7.28			S.E.**	
Initial controls	11	21.65	5.29	4—acetone	8	0.13±0.28	10%
Conclusion controls	17	16.16	0.19	40—acetone	8	1.36±0.27	100%
Acetone	13	21.19	0.05	40—0.5% TCA	10	0.02±0.39	0%
0.1% TCA	14	17.15	0.27	40—2.0% TCA	10	1.20±0.20	85%
2.5% TCA	14	17.24	0.14	40—2.5% TCA	10	1.42±0.38	100%
10.0% TCA	12	21.89	0.44				

* Hypox controls were killed 24 hours after hypophysectomy, whereas the initial controls were killed on the day the injections were begun, 12 days later.

** Standard errors include the standard error of the zero potency values obtained from adrenal ascorbic acid levels of acid saline diluent injections.

† Calculated sample variances in which S = standard deviation.

within "basophiles." Table 3 indicates that in the present work FSH, LH and TSH are completely absent in 0.5% TCA extracted pituitary glands. It confirms the fact that LH is precipitated by 2.5% TCA and also establishes that FSH and TSH are not completely precipitated by even 10% TCA. In addition to the weight data presented in Table 3, the histological appearance of the ovaries of the recipient hypophysectomized rats was examined in the assays concerned with LH and FSH. The ovaries of assay animals injected with pituitaries extracted with 0.5% TCA appeared the same as those of uninjected hypophysectomized controls. Injection of pituitaries extracted in 2.5% TCA caused a remarkable increase in the amount of interstitial tissue, but the follicles largely remained similar to those in the ovaries of the conclusion controls, except for the luteinization of a few. Follicle size and development increased somewhat over that of the hypophysectomized level after injection of pituitaries extracted in 10% TCA, but Graffian follicles were never seen in the ovaries of any of the recipient hypophysectomized rats.

TABLE 3. BIO-ASSAY OF UNEXTRACTED AND EXTRACTED RAT PITUITARY GLANDS FROM NORMAL ANIMALS INDICATING GONADOTROPIN AND THYROTROPIN CONTENT

	No. of animals	Mean gain in body wgt. (gm.)	Mean ovary wgt. ±S.E. (mg.)	Mean uterine wgt. ±S.E. (mg.)	Mean testis wgt. ±S.E. (mg.)	Mean seminal vesicle wgt ±S.E. (mg.)	Mean prostate wgt.±S.E. (gm.)	Mean thyroid acinar cell height in μ	
								No.	±S.E.
Initial controls	8M 4F	— —	11.6±1.3	20.3±2.2	263.7±15.2	6.8±0.5	15.7±0.7	5	3.25±0.07
Conclusion controls	10M 9F	6 3	10.7±0.09	18.3±0.7	168.8± 8.0	5.9±0.3	5.9±0.5	5	2.53±0.21
Acetone/dried pituitary	6M 6F	13 6	71.9±5.6	97.6±6.4	423.4±25.4	20.9±1.7	23.9±1.2	4	3.79±0.30
Extracted 10% TCA	6M 5F	10 8	39.9±3.8	36.2±3.8	312 ±18.6	16.6±1.2	21.7±1.0	5	2.69±0.20
Extracted 2.5%	8M 8F	7 8	12.7±1.0	17.5±1.7	251 ± 7.1	11.1±0.6	18.3±1.3	8	2.67±0.15
Extracted 1% TCA	6M 6F	6 6	11.3±1.1	16.4±1.4	187.4±10.4	6.5±0.4	7.9±0.7	5	2.50±0.09
Extracted 0.5% TCA	6M 6F	6 5	11.1±1.0	17.2±1.5	182.0± 9.2	6.3±0.4	7.1±0.7	5	2.48±0.08

Cytology of the Normal Extracted and Unextracted Anterior Pituitary Glands. In the normal rat pituitary gland the granular cytoplasm of acidophils exhibits a strong reaction for SH and S-S groups and for α-acylamido carboxyl groups (Fig. 1). Although under high power of magnification with the light microscope there was some degree of difference of intensity of staining of individual cells with either method from moderate to intense, these differences were neither regional nor could they be assigned to round, oval, polygonal or columnar-shaped acidophils. Cells that reacted most intensely with one method also reacted with equal intensity with the other and the intensity of reaction appeared to correlate well with the content of granules in the cytoplasm. Proof that acidophils were staining with these methods depended not only on the recognition of their morphological characteristics but on the successful comparison of the same cells in parallel 3 μ sections, one stained with one of the histochemical methods and the other with Orange G. Basophils (PAS-positive cells) and chromophobes stained weakly or not at all with the methods for SH and S-S and for α-acylamido carboxyl groups. In preliminary experiments pituitaries were stained also by a specific method for amino groups of protein (17). Although the acidophils reacted more than the other cell types with this method, they were less reactive than with the histochemical methods finally used.

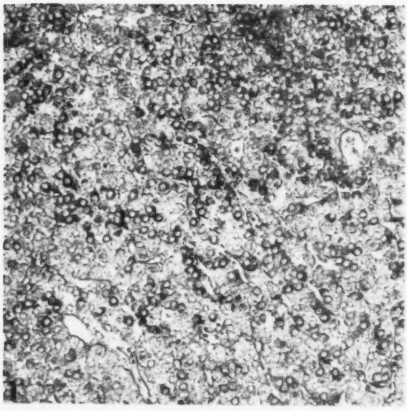
Normal pituitaries that had been extracted in 0.5% TCA, refixed and stained by the above methods showed entirely different results in comparison to controls. Under these circumstances less than 10% of the original acidophil population were reactive; the remainder of the parenchymal cells were completely unstained (cf. Figs. 1 and 2). The reactive acidophils were

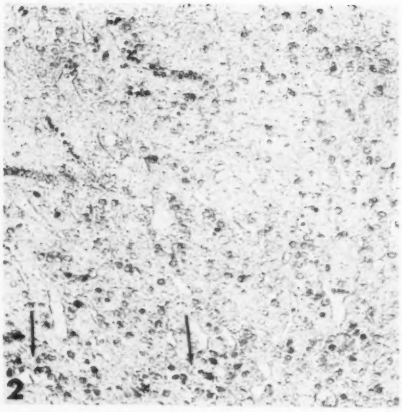
All photomicrographs are taken of sections of rat pituitary gland stained for the carboxyl groups of protein. Identical results are obtained with the method for sulfhydryls and disulfides. Figures indicate the differences between unextracted (odd numbers) and extracted (0.5% TCA) glands (even numbers).

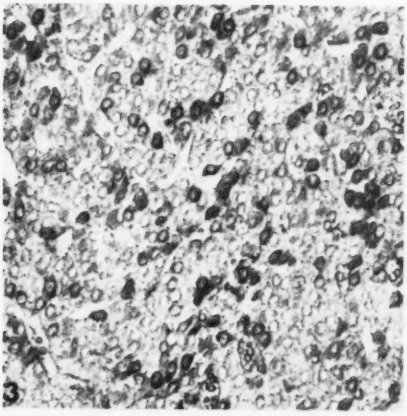
FIGS. 1 and 2. Low power photomicrographs indicating the distribution of histochemically reactive acidophils in the pituitary glands of a normal, female estrus rat. The reactive cells of the unextracted gland are intensely or moderately stained (Fig. 1). In the extracted pituitary gland (Fig. 2) the number of reactive cells (some indicated by arrows) is vastly decreased. Furthermore, the cells are shrunken and distorted. Most of the stained elements at the top of photomicrograph are blood cells within sinusoids. $\times 100$.

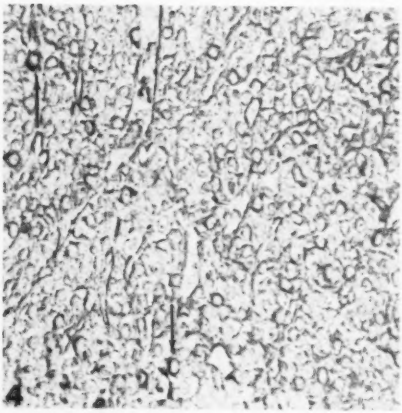
FIGS. 3 and 4. Photomicrographs of sections of pituitary glands of rats which were pregnant for 10 days. The unextracted one (Fig. 3) indicates the distribution of acidophils by the carboxyl method but, in comparison, the extracted gland that contains only LTH shows that only few acidophils (arrows) are reactive. $\times 200$

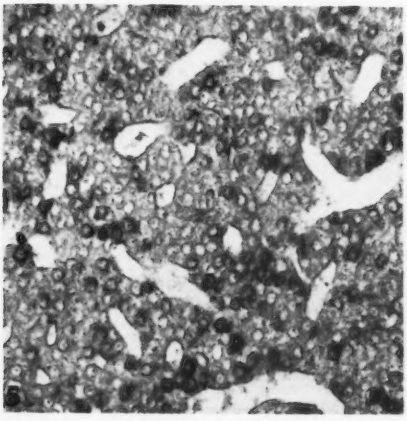
FIGS. 5 and 6. Photomicrograph of sections of pituitary glands of lactating rats taken two days post-partum. Comparison of these glands indicated that it is difficult to distinguish between the extracted (Fig. 6) and control (Fig. 5) gland. This would indicate that most of the acidophils contained LTH at this time. $\times 200$

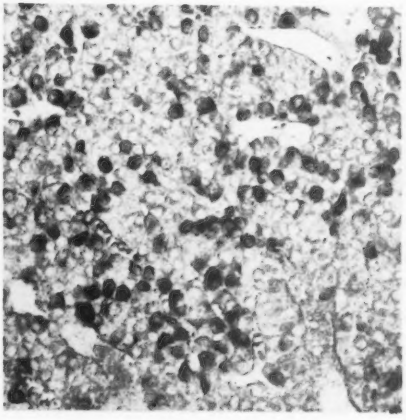












more intensely stained than those in unextracted glands but they were also more shrunken and distored in shape, presumably due to their previous treatment. Although the reactive cells were not sharply restricted in their localization, they tended to occur more commonly in an area intermediate between the periphery and the central region of the gland. Pituitaries that had been extracted in 0.05 or 0.1% TCA, on the other hand, contained no cytoplasmic reaction in any of the parenchymal cells stained with any of the methods, although some cells of the stroma as well as the basement membrane surrounding the sinusoids were weakly reactive with the histochemical methods. It should be noted here that the number of acidophils that stained in pituitaries after 2.5% TCA extraction was greatly increased over those extracted in 0.5% TCA and after extraction in 4% TCA the number of reactive acdophils and their intensity of reaction appeared the same as those in unextracted glands.

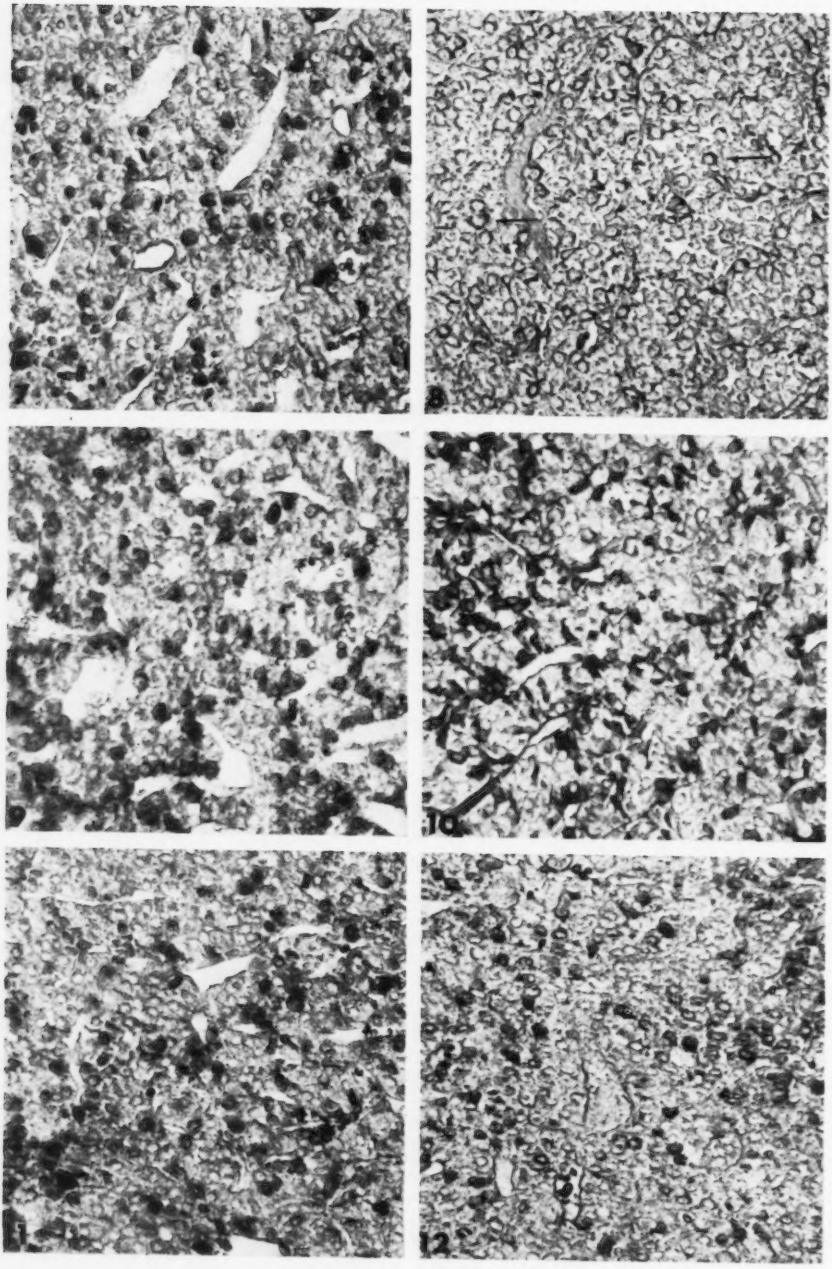
Pituitary Glands of Pregnant and Lactating Rats. It appeared warranted in order to complete our argument to examine pituitary glands of rats in which the LTH content had been increased. Therefore, as Everett and Baker (18) had done previously in mice, we examined the stained, extracted and unextracted glands of pregnant and lactating rats. For these purposes, instead of performing assays on these animals, we were guided by the results of Hurst and Turner (19) who reported the LTH content per mg. of pituitary tissue of various species including rats at various stages in pregnancy and during lactation. Our results proved to provide a morphological counterpart of those reported by Hurst and Turner (19). Pituitary glands taken at the 10th day of pregnancy and extracted in 0.5% TCA showed little or no change from those of extracted normal female pituitaries as concerns the number of acidophils and the intensity with which they stained (cf. Figs. 4 and 2). At the 15th day of pregnancy, the extracted glands showed a slight increase in the number of reactive acidophils (cf. Figs. 4 and 8). At both the 10th and the 15th day of preganancy, most of the acidophils did not contain LTH (cf. Figs. 3 with 4 and 7 with 8).

On the other hand, 0.5% TCA extracted pituitaries taken from rats within a few hours of parturition showed an obvious increase in the number of acidophils and the intensity with which they stained. This was much more marked at two or three days postpartum, the time of peak LTH production (cf. Figs. 6 and 10 with 2 or 4). In these pituitaries not only was the acidophil population the highest and the intensity of histochemical staining greatest, but it was difficult to distinguish between the stained sections of unextracted and extracted glands (cf. Figs. 5 with 6 and 9 with 10). This finding indicated that almost the entire acidophil population contained LTH at this stage. Extracted pituitaries taken from rats which had been lactating for 7 days showed a decrease in the number of cells that stained as well as the intensity with which they stained (cf. Figs. 6 or 10 with 12) and not all the acidophils contained LTH (cf. Figs. 11 and 12).

DISCUSSION

The present experiments which combine the use of several techniques relating to the differential solubilities of proteins, histochemical staining and bio-assay, demonstrate unequivocally that LTH is present in the granular cytoplasm of the acidophils in the rat anterior pituitary gland. Indeed, these experiments provide a method for demonstrating LTH to the exclusion of other anterior pituitary hormones since it was shown that LTH was present in normal amounts in the extracted and stained glands; that the staining varied directly with the amount of hormone present in different physiological states; and that the other anterior pituitary hormones were completely absent by bio-assay. The method presented has several virtues. The first of these is the use of fixation in a specific way so as to take advantage of one of the most delicate properties of proteins, that is, differential solubility. Secondly, specific histochemical methods (15, 16) that can be quantitated were used instead of selective and sometimes vicarious histological methods. Finally, bio-assays of the extracted and unextracted glands provided a measurable base-line of function upon which the morphological data could be directly interpreted. Although Gersh demurs (20), specific histochemical staining coupled with specific extraction provides a methodology that can be generally extended for the demonstration of protein hormones. In fact, since its introduction by Catchpole (21) and Gersh (22), we have successfully used this principle, in part, for the demonstration of neurohypophyseal hormones (23), insulin (24), and glycoprotein hormones of the adenohypophysis (1). The principle appears also to be adaptable for other protein hormones of the hypophysis and placenta (2), and has been widely used for the demonstration of muscle proteins (25).

It should be pointed out that the staining methods used and our working hypothesis that the pituitary simple protein hormones of the rat are in acidophils, tend to force a circular argument for the following reasons: First, after dissolution of the glycoprotein hormones, but not all of the simple protein hormones, basophils and not acidophils are virtually empty of cytoplasmic granular material and are therefore unstained by any method (1). Secondly, there are no specific methods for the histochemical demonstration of the class of simple protein hormones of the pituitary as there are for the glycoproteins with the Periodic-acid-Schiff (PAS) method and appropriate controls. These facts lead us to use specific methods for groups in proteins but these methods must be considered general for proteins including the simple protein hormones unless the latter have a preponderance of these groups. However, we already knew (15, 16, 26, 27) that when these methods were applied to appropriately fixed sections of normal, unextracted pituitary glands that only the granular cytoplasm of the acidophils stained intensely. Despite this hazardous approach from a logical point of view, we felt that significant information could be gained from the solubility studies that would either reinforce these histochemical findings or



negate them depending on whether any histochemical changes were produced on dissolution of one or another of the simple protein hormones.

Experimental circumstances that alter the ACTH or STH content in the gland also alter the number of acidophils that stain by these methods and/or change the intensity of their staining (2, 3, 27). It may be of significance in this regard that use of high concentrations of TCA that precipitated both ACTH and STH caused an increase in the number of histochemically reactive acidophils to the level of that of the unextracted control glands. In the present experiments, the SH and S-S method was used to demonstrate only S-S groups, since it is known that the content of these groups in LTH is high and that there are no free SH groups in the anterior pituitary hormones (10). The fact that an intense reaction occurred for α-acylamido carboxyl groups suggests that LTH probably contains a large number of side chain carboxyls such as those of glutamic acid (28), rather than being a small molecular weight protein.

Earlier workers indicated that changes in the acidophil population and granulation accompany pregnancy and lactation (29–33). More recently, when knowledge of the hormone became substantial, many more regarded the acidophil as the site of production of LTH (18, 34–41). These works were performed on a variety of species including pigeon (34), rodents (18, 41), cat (36) and cow (35). Furthermore, these experiments studied a variety of experimental conditions, including pregnancy and lactation (18), post-coitus (36), anterior pituitary autografts (37) and lactogenic tumors produced by large doses of estrogens (38), in which changes occurred in the pituitary content of LTH as well as the acidophil population. Indeed, Herlant (39) obtained granules by differential centrifugation of pituitary homogenates which were identified as acidophilic in character and con-

All photomicrographs (×200) were taken from rat pituitary glands stained for carboxyl groups of protein. Figures indicate the difference between unextracted (odd numbers) and extracted (0.5% TCA glands (even numbers)).

FIGS. 7 and 8. Photomicrographs of sections of pituitary glands of rats which were pregnant 15 days. The section of the extracted gland (Fig. 8) shows that only few cells are histochemically active (arrows) in comparison to the section of an unextracted gland (Fig. 7). However, there are more cells which show activity than in the glands of 10-day pregnant rats (cf. Fig. 4).

FIGS. 9 and 10. Photomicrographs of sections of pituitary glands of lactating rats taken 3 days post-partum. At this stage, the peak of LTH storage in the pituitary, there is virtually no difference between the extracted (Fig. 10) and unextracted glands (Fig. 9).

FIGS. 11 and 12. Photomicrographs of sections of pituitary glands of lactating rats taken 7 days post-partum. Comparison indicates that the unextracted gland (Fig. 11) contains more reactive cells than the extracted gland (Fig. 12). In addition, the number of reactive cells in the extracted gland has decreased from the 3-day post-partum level (cf. Fig. 10).

tained crop-stimulating activity. The present work adds to those cited above a degree of histochemical specificity so that there can no longer be a question as to the site of origin of LTH.

Although the present experiments do not present firm evidence for the existence of LTH in a separate sub-class of acidophils, such is felt to be the case. If all the simple protein hormones were mixed in the acidophils, extraction of two of them would have produced a decrease in the intensity of staining. This was not the case, however, in the extracted control glands. Only a decrease in the number of acidophils that stained was evident and those showed a reaction that was of usual intensity. The great increase in the number of reactive acidophils present in the extracted pituitary glands of lactating rats can be attributed to the possibility of other, normal unreactive acidophils had taken over an LTH function. However, this does not seem likely since it has been shown that after parturition there is a greatly increased mitotic activity in acidophils (18, 42) so that their number in the pituitaries of lactating rats at 3 days postpartum increases almost 100% above the number present in glands of non-lactating pregnant animals. The percentage increase is even greater when the comparison is made with the glands of normal non-pregnant animals. The probability of a subclass of acidophils concerned with the production of LTH must be taken in correlation with the fact that several selective histological methods that use a number of acid dyes, show more than one tinctorially different types of acidophils (43–45). Moreover, in several works one of these tinctorial cell types has been directly implicated in the production of LTH (36, 38, 41).

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SPONTANEOUS SODIUM CHLORIDE APPETITE OF GOITROGEN-TREATED RATS: EFFECT OF HYPOPHYSECTOMY AND ADRENALECTOMY[1]

M. J. FREGLY, O. GALINDO AND K. M. COOK

Department of Physiology, University of Florida, College of Medicine, Gainesville, Florida and Department of Biology, Coe College, Cedar Rapids, Iowa

ABSTRACT

Administration of the anti-thyroid drugs, propylthiouracil, thiouracil and methimazole, is accompanied by an increase in the spontaneous NaCl intake of male and female rats offered choice between water and .15 M NaCl solution to drink. The increase in NaCl intake occurs within one week after treatment begins and is independent of either pituitary or adrenal glands.

A SPONTANEOUS NaCl appetite may be measured behaviorally by allowing rats free choice between a NaCl solution and water as drinking fluids. At least three experimental procedures are known to affect appetite for salt: (a) renal and metacorticoid hypertensions decrease it below that of controls (1–3), while (b) bilateral adrenalectomy and (c) administration of desoxy-corticosterone-acetate increase it (4–7). The mechanisms are unknown. Preliminary studies suggested that administration of the anti-thyroid drug, propylthiouracil, abolished the spontaneous NaCl aversion of renal hypertensive rats (8). A primary objective of the present experiments was to study the effect of treatment with anti-thyroid drugs on spontaneous NaCl intake of "normal" rats. When it was established that these rats exhibited a NaCl appetite, further objectives were to determine whether either pituitary or adrenal glands played a role.

METHODS AND RESULTS

Five separate experiments were performed. Each is described below.

The object of the first experiment was to study the spontaneous NaCl intake of intact male and female rats treated with the anti-thyroid drug, propylthiouracil (PTU),[2] administered at a dose level of 0.1 gm. per 100 gms. of food. Ten male and 8 female rats were used. The rats in each group were born within 2 days of each other. Five of the male rats had received PTU during 18 weeks prior to beginning the experiment while 4 female rats had received PTU during 9 weeks. All rats were kept in individual cages in a room maintained at $26 \pm 1°$ C and illuminated from 8 A.M. to 6 P.M. Intakes of food (ground Purina chow), distilled water, and .15 M NaCl solution, were measured for 1 week, using a triple beam balance for measurement. Food and fluid containers used are described in detail elsewhere (2, 9).

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[2] 6-n-propyl-2-thiouracil, Nutritional Biochemicals Corp., Cleveland, Ohio.

PTU-treated male and female rats had significantly greater NaCl solution intakes relative to body weight than control rats (Table 1). Water intakes were not affected by PTU treatment; however, total fluid intakes (water+NaCl solution) were increased significantly ($P < .01$). Food intakes of both groups of PTU-treated rats were less than those of controls. The difference was significant only in the case of the male rats. Mean body weights of PTU-treated rats were less than those of corresponding controls although initial body weights at the beginning of the experiment were the same. This difference in body weight is due to the well-known, growth-inhibiting effect of propylthiouracil.

TABLE 1. THE EFFECT OF PROPYLTHIOURACIL ON SPONTANEOUS FLUID AND FOOD INTAKES OF MALE AND FEMALE RATS

Treatment	No. of rats	Mean body weight (gms.)	Intakes (ml. or gm./100 gm. BW/Day)		
			Water	.15M NaCl Soln.	Food
Male Rats					
Control	5	458	6.0±1.5[1]	1.8±0.4	4.4±0.3
PTU-Treated[2]	5	406	6.5±0.6	8.7±1.0*	3.0±0.2*
Female Rats					
Control	4	260	5.6±0.9	13.3±1.8	7.2±0.6
PTU-Treated[3]	4	243	5.1±0.9	21.9±2.4*	6.2±0.7

[1] ±1 standard error of mean.
[2] Given PTU for 18 weeks prior to experiment.
[3] Given PTU for 9 weeks prior to experiment.
All intakes were measured for 1 week.
* Significantly ($P < .01$) different from control.

The second experiment extended the results of the first to include the drugs thiouracil[3] and methimazole.[4] Ten male Holtzman rats were used in the first series (thiouracil) and 8 in the second (methimazole). Half of the rats in each series was given the drug mixed into food (Purina Chow) at levels of 0.1 and 0.05% respectively. All rats were caged individually under the same conditions described in Experiment 1. The same choice of drinking fluids was offered for 2 weeks. All treated rats had been given the drug during 8 weeks prior to the experiment.

Both thiouracil- and methimazole-treated rats had a significantly greater salt intake than that of their controls (Table 2). Total fluid intake relative to body weight was greater in both treated groups than in corresponding controls. Food intake of thiouracil-treated rats was slightly less than that of controls, while food intake of methimazole-treated rats was unaffected. Again, body weights of the treated groups were less than those of corresponding controls. The results of this experiment confirm those observed in the first experiment.

The third experiment was conducted to determine the time course of development of the salt appetite after goitrogen administration. Twenty male Holtzman rats were used. After a 2-week control period the rats were

[3] 2-thiouracil, Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] methimazole (Tapazole®), courtesy of Dr. Glenn Irwin, Eli Lilly and Co.

TABLE 2. EFFECT OF THIOURACIL AND METHIMAZOLE ON SPONTANEOUS FLUID AND FOOD INTAKES OF RATS IN EXPERIMENT 2

Treatment	No. of rats	Mean body weight (gms.)	Intakes (ml. or gm./100 gm. BW/Day)		
			Water	.15M NaCl Soln.	Food
Control	5	440	9.2±0.8[1]	2.6±0.5	4.8±0.1
Thiouracil-Treated[2]	5	305	10.4±0.6	6.8±0.9*	4.6±0.2
Control	4	515	5.1±0.5	3.2±0.5	3.7±0.1
Methimazole-Treated[3]	4	427	3.3±0.5	9.2±0.8*	3.8±0.3

[1] ±1 standard error of mean.
[2] 0.1% in diet.
[3] 0.05% in diet.
* Significantly ($P < .01$) different from control.

divided at random into groups of 5. One group served as control, while the remaining 3 groups were given 0.2% propylthiouracil, 0.2% thiouracil and 0.2% methimazole in the ground Purina chow diet. Intakes of water and NaCl solution were measured daily during the control period and for an additional 13 weeks during drug administration. Fig. 1A illustrates that an

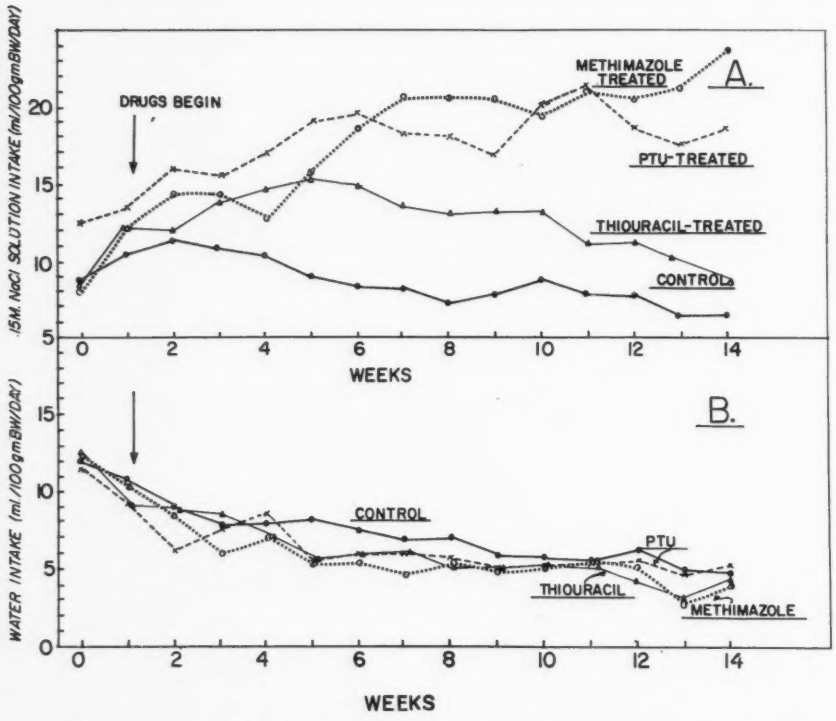

FIG. 1 A. Intake of .15 M NaCl solution of control (●), thiouracil-treated (Δ), PTU-treated (×), and methimazole-treated (○) rats throughout the 14 weeks of the experiment. B. Intakes of water for the same groups as in A.

ncrease in salt appetite is apparent within 1 week after treatment begins or propylthiouracil- and methimazole-treated rats and within 2 weeks for hiouracil-treated rats. The level of NaCl solution ingested by treated rats emained higher than that of controls throughout the experiment. Thioura-il-treated rats showed the least response. Their intake of NaCl solution began to wane after the 5th week of treatment and nearly reached control levels by the end of the experiment. Water intakes of the treated rats were generally slightly less than that of controls throughout the experiment (Fig. 1B).

The fourth experiment was composed of 3 separate but similar experimental parts. The purpose of each was to determine whether PTU treatment influenced the NaCl intake of male hypophysectomized rats. PTU was administered at a level of 0.1% in the diet. Intakes of distilled water, .15 M. NaCl solution and food were measured daily for 7 to 10 days in the 3 experimental parts. The first 2 used 10 rats while the last used 8. Half of the rats in each experimental part received food supplemented with PTU. The groups had been hypophysectomized for different lengths of time, although all had been hypophysectomized at 28 days of age. The groups also had received PTU for different lengths of time when each experiment began. The time after hypophysectomy and duration of PTU treatment are given in Table 3 for the rats in each experimental part.

In all 3 experimental parts, PTU increased the NaCl intake of hypophysectomized rats, although the increase was significant only in the first and third parts (Table 3). Neither water intake nor food intake was influenced by PTU treatment. The latter observation is contrary to that seen in intact rats (10). Thus, the spontaneous NaCl appetite accompanying PTU treatment does not depend on an intact pituitary gland.

A fifth experiment was performed to determine whether the adrenal glands participated in the spontaneous NaCl appetite of PTU-treated rats. Ten male Holtzman rats were used. They were divided into 2 groups of 5. All rats were given choice between .15 M. NaCl solution and water to drink for 10 days. Ground Purina chow was used as

TABLE 3. EFFECT OF PROPYLTHIOURACIL ON SPONTANEOUS FLUID AND FOOD INTAKES OF HYPOPHYSECTOMIZED RATS

	Number of rats	Mean body wt. (gms.)	Water intake (ml./100 gm. BW/Day)	.15M NaCl intake (ml./100 gm. BW/Day)	Food intake (gm./100 gm. BW/Day)
Experiment 1[1]					
Hypox+Chow	5	107	16.4±0.7[4]	4.4±0.5	7.9±0.4
Hypox+0.1% PTU	5	96	17.9±1.1	11.2±1.7*	8.1±0.4
Experiment 2[2]					
Hypox+Chow	5	125	10.2±0.5	8.1±0.9	6.0±0.3
Hypox+0.1% PTU	5	104	9.2±0.8	10.7±1.3	5.7±0.4
Experiment 3[3]					
Hypox+Chow	4	152	8.4±0.7	4.3±0.5	5.9±0.5
Hypox+0.1% PTU	4	130	9.2±0.7	6.9±0.6*	6.5±0.9

[1] Hypophysectomized 6 weeks prior to experiment; given PTU 4 weeks prior to experiment; intakes measured for 7 days.

[2] Hypophysectomized 14 weeks prior to experiment; given PTU 12 weeks prior to experiment; intakes measured for 10 days.

[3] Hypophysectomized 16 weeks prior to experiment; given PTU 14 weeks prior to experiment; intakes measured for 10 days.

[4] ±1 standard error of mean.

* Significantly ($P < .01$) different from controls.

food for both groups. Food and fluid intakes and body weights were measured daily. At the end of the 10-day control period, the first group was given chow containing 0.1% PTU thoroughly mixed into it. Measurement of intakes continued for 14 days at the end of which *both* groups of rats were adrenalectomized. After recovery, intake measurements continued for 14 more days. The experiment was performed in this fashion because prior experience had taught that adrenalectomized rats given 0.1% PTU in food die within 1 week. However, if intact rats are given PTU for 2 weeks and then adrenalectomized, they will survive.

It may be observed from Fig. 2A that both NaCl solution and water

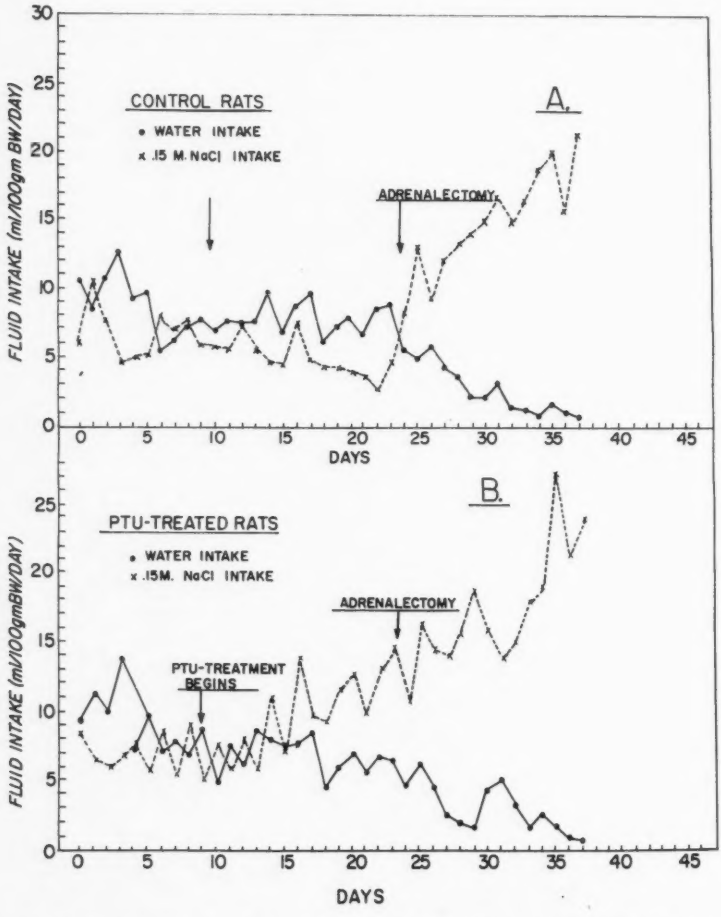

FIG. 2. A. Mean intakes of water (●) and .15 M NaCl solution (×) for 5 control rats throughout the experiment. These rats were adrenalectomized on the 24th day of the experiment. B. Mean intakes of water (●) and .15 M NaCl solution (×) for 5 PTU-treated rats throughout the experiment. PTU treatment began on the 9th day of the experiment. All rats were adrenalectomized on the 24 th day of the experiment.

intake of control rats gradually diminished during the first 21 days of the experiment. This is probably due both to growth of the rats and to calculation of fluid intake on a body-weight basis (ml./100 gm. BW/day) (10). Fluid intake apparently does not increase in direct proportion with increasing body weight. In this group, NaCl intake was generally somewhat less than water intake. After adrenalectomy on the 23rd day of the experiment, intake of NaCl solution increased sharply and continued to increase for the next 2 days. After adrenalectomy, intake of salt solution far exceeded water intake. In the PTU-treated group (Fig. 2B), intake of NaCl solution increased within 1 week after PTU treatment began, finally reaching levels higher than those observed for adrenalectomized controls during this period.

Table 4 gives the water, .15 M. NaCl solution, and food intakes of both groups of rats in the 5th experiment during the last 5 days of each treatment. During the last 5 days of the control period, all intakes of the two groups were similar. During the last 5 days of the period of PTU treatment, intake of NaCl solution increased significantly above level of control rats while food intake decreased significantly. During the last 5 days of the

TABLE 4. FOOD AND FLUID INTAKES (ML. OR GM./100 GM. BW/DAY) OF THE TWO GROUPS OF RATS IN EXPERIMENT 5 DURING THE LAST 5 DAYS OF EACH EXPERIMENTAL PERIOD

Day	Control (5)			Experimental (5)		
	Water	.15M NaCl		Water	.15M NaCl	
		Solution	Food		Solution	Food
Control Period						
5	9.6	5.1	7.6	9.7	5.8	8.1
6	5.5	9.5	7.3	7.1	8.5	7.4
7	6.1	8.7	7.4	7.9	5.8	7.0
8	7.1	7.7	7.8	6.9	9.1	8.2
9	7.8	5.9	6.9	8.6	5.2	7.1
	7.2 ±0.9	7.4 ±1.0	7.4 ±0.1	8.0 ±0.8	6.9 ±1.2	7.6 ±0.2
PTU Treatment (Experimental Group Only)						
19	7.1	4.2	5.9	6.1	11.8	4.2
20	7.8	3.8	6.6	7.0	12.8	5.8
21	6.6	3.7	6.1	5.8	10.0	5.1
22	8.5	2.7	6.7	6.9	13.1	6.3
23	8.7	4.7	6.1	6.5	14.8	6.1
	7.7 ±0.7	3.8 ±0.6	6.3 ±0.1	6.5 ±0.8	12.5 ±1.5*	5.5 ±0.2*
Adrenalectomy (Both Groups)						
33	1.3	16.4	5.5	1.9	18.2	5.1
34	0.9	18.8	5.5	2.8	19.3	5.7
35	1.6	20.0	5.6	2.0	27.4	6.1
36	1.5	15.7	4.6	1.2	21.4	2.3
37	0.8	21.4	5.4	1.0	24.3	5.2
	1.2 ±0.2	18.5 ±0.9	5.3 ±0.2	1.8 ±0.2†	22.1 ±1.3†	4.9 ±0.3

* Highly significant difference from control ($P < .01$).
† Significantly different from control ($P < .05$).

period of combined adrenalectomy and PTU treatment, intakes of both NaCl solution and water were significantly greater for the PTU-treated, adrenalectomized rats than for that of controls which were adrenalectomized only. Food intakes of the two groups were similar.

Statistical analyses of the data from each experiment were performed to compare simultaneous means using the "t" test for the 95% confidence limit (11).

DISCUSSION

When choice was offered between water and .15 M. NaCl solution to drink, rats treated with the anti-thyroid drugs, propylthiouracil, thiouracil and methimazole, generally ingested more NaCl solution than water. They always ingested more NaCl solution than untreated controls. The fluid intakes are expressed as ml./100 gms. BW/day in this study. It may be argued that the lower body weight of the goitrogen-treated rats exaggerates the difference between fluid intakes of treated and control rats. However, all rats used were matched initially for body weight and age. Since age was controlled, it seemed fair to correct fluid intakes to a standard body weight and thus compare treated with control rats. In normal rats, body weight is known to be an important correlate of water exchange (12). We assume this to be so in the hypothyroid state. In the case of hypophysectomized rats, where differences in body weight between treated and untreated rats are less than those observed for intact rats, the increased intake of NaCl solution by treated rats still persists (Table 3).

The increase in intake of NaCl solution observed to accompany goitrogen administration occurs within one week after initiation of treatment. It is independent of the pituitary gland since PTU-treated, hypophysectomized rats also manifested an increase in intake of NaCl solution above that of untreated hypophysectomized rats (Table 3).

A question naturally arises concerning the participation of the adrenal glands in the goitrogen-mediated salt appetite. Hence, it was important to assess the effect of PTU on intake of salt solution by adrenalectomized rats. The results of these experiments indicate that the increased intake of salt solution by PTU-treated rats is independent of the adrenal glands since PTU-treated, adrenalectomized rats had a greater intake than that resulting from adrenalectomy alone (Table 4).

These experiments have excluded the possibilities that pituitary and adrenal glands play a role in the goitrogen-induced salt appetite. It is not known at present whether these drugs act at the level of the kidney tubule to decrease sodium reabsorption and increase sodium loss, thereby stimulating salt appetite. These drugs increased total fluid (water+NaCl solution) intake (Tables 1 and 3) in these experiments as well as in earlier experiments where water was the sole drinking fluid (13). The increased fluid intake is attributed to increased urinary flow resulting from both a

diminished kidney response to anti-diuretic hormone and from possible kidney tubular effects of the drugs similar to those produced by the aminouracils. The chemical structure of the latter is similar to that of thiouracil and propylthiouracil (14). Whether the increased urinary flow is accompanied by increased urinary sodium and chloride loss is also not known at present. The effect of goitrogens on urinary loss of sodium and potassium is currently under study.

Acknowledgments

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THE ANTI-FERTILITY EFFECTS IN PREGNANT RATS OF A SINGLE INJECTION OF ESTRADIOL CYCLOPENTYLPROPIONATE

GILBERT S. GREENWALD[1,2]

Department of Anatomy, University of Washington, Seattle, Washington

ABSTRACT

Sprague-Dawley female rats were injected subcutaneously with varying dosages of estradiol cyclopentylpropionate (ECP) on the morning that sperm were found in the vagina. The percentage of embryos missing at 9–11 days *post coitum* after a single injection of 1.5 or 10 μg of ECP was 19%, 66%, 97%, respectively.

Deciduomata formation was inhibited in most animals that received 10 μg of ECP but was unaffected in those receiving 1 or 5 μg. The most significant effect of 10 μg ECP was to increase tubal and uterine motility so that the fertilized ova were expelled from the uterus by 48 hours *post coitum*. The effectiveness of 5 μg of ECP in accelerating ova transport varied from animal to animal. These results indicate that the rate of ova transport is an especially vulnerable process in the reproductive cycle for interrupting pregnancy in the rat.

THE effectiveness of estrogens in interrupting pregnancy in the rat and mouse was first shown by Parkes and Bellerby (1) and Smith (2) in 1926. This finding initiated a very active and fruitful period of research during the following decade (for references consult 3 and 4). Recently, there has been renewed interest in the effects of exogenous estrogens on pregnancy (5–11). In most of these studies, the anti-fertility effects of estrogens administered before implantation were judged by the number of young born alive or dead. This method, while essential as an initial procedure, is of little value in determining the mechanisms that are affected by the hormonal treatment.

It has been reported recently that ova transport in the rabbit is accelerated or retarded after the injection, respectively, of 25 or 250 μg of estradiol cyclopentylpropionate (ECP) (12). These findings may offer a new approach to fertility control and prompted the present investigation. The study to be reported here has two major aims: 1. to establish the minimum effective dosage of ECP required to interrupt early pregnancy in the rat and 2. to determine the mechanisms involved in embryonic death during this period.

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[2] Present address: University of Kansas Medical Center, Kansas City, Kansas

MATERIALS AND METHODS

A total of 93 Sprague-Dawley female rats weighing 240–290 gms. were used. Females were caged with males of proven fertility and on the morning that sperm were found in the vaginal smear (designated as day 1 of pregnancy) the females were injected s.c. with either 1, 5, or 10 μg of ECP. The 1 and 5 μg dosages were injected in a volume of 0.1 ml. of cottonseed oil, the higher dosage in 0.2 ml. of the vehicle. Twenty-four animals treated with ECP or 0.1 ml. of cottonseed oil (control group) were killed between 9 and 11 days after mating and the number of embryos and corpora lutea counted (Table 1). The large vascular corpora lutea of pregnancy were distinguished from the regressing corpora lutea of ovulation by the use of a 25×stereoscopic binocular dissecting microscope.

After establishing the dosage of ECP necessary to interrupt pregnancy, the next step was to study the physiologic mechanisms that were affected by the hormone. To establish the effects of estrogen on *ova transport*, 29 rats were injected on day 1 of pregnancy with 5 or 10 μg of ECP. The animals were killed at varying intervals during the preimplantation period by an overdose of ether. The oviducts and cornua were flushed sepa-

TABLE 1. THE EFFECTS OF A SINGLE INJECTION OF ECP ON RAT PREGNANCY*

Treatment	No. animals	No. pregnant	Mean No. corpora lutea (±S.E.)	Mean No. embryos (±S.E.)	% embryos lost
0.1 ml. cottonseed oil	4	4	13.5±0.96	10.5±2.9	22.2
1 μg. ECP	4	4	13.8±0.75	11.3±2.8	19.1
5 μg. ECP	8	4	11.3±0.59	3.9±1.7	65.5
10 μg. ECP	8	1	13.0±0.69	0.4±0.37	96.9

* Rats killed 9–11 days *post coitum*.

rately with physiological saline by a 30 gauge needle attached to a 1 ml. syringe. The recovered ova were counted and compared with the number of corpora lutea (Table 2).

To determine the effects of estradiol cyclopentylpropionate on *the endometrium*, 4 groups of animals (each consisting of 5 rats) were injected on day 1 with either 0.1 ml. cottonseed oil or 1, 5 or 10 μg of the hormone. The animals were anesthetized with ether on day 5 and the uterus exposed by a mid-abdominal incision. The endometrium of the right cornu was traumatized along its entire antimesometrial border by a barbed needle inserted into the lumen at the level of the corpus uterus. The left uterus was not traumatized and served as a control. The animals were killed 72 hours later; the uteri were stripped of fat and weighed immediately on a torsion balance.

RESULTS

The effects of ECP on pregnancy. The injection of 1 μg of ECP had no more effect on pregnancy than 0.1 ml. of cottonseed oil (Table 1). However, after the injection of 5 μg of ECP 4 of 8 animals were later found to be pseudopregnant and only 1 of the remaining pregnant rats was completely unaffected. The administration of 10 μg of ECP caused a marked reduction in the pregnancy rate in a group of 8 animals. One female had 2 embryos when killed at 10 days *post coitum;* the other seven were pseudopregnant (Table 2).

The effects of ECP on ova transport. After the injection of 5 μg of ECP, there was considerable variation from animal to animal in the number of

TABLE 2. THE EFFECTS OF ECP ON OVA DISTRIBUTION

Treatment	Time examined (Days P.C.)	Female No.	No. corpora lutea	No. ova recovered	Location of ova	Remarks on ova
5 μg ECP	3	1	14	14	Oviduct	2–3 celled
		2	14	11	Oviduct	4 celled
		3	13	7	Oviduct	2 celled
		4	11	5	Oviduct	2 celled
		5	11	1	Oviduct	4 celled
		6	10	0	—	—
		7	9	0	—	—
			$\bar{x}=11.6$	$\bar{x}=5.4$		
	4	8	14	5	Uterus	Morulae
	5	9	12	6	Uterus	Blastocysts
		10	14	6	Uterus	Blastocysts
		11	12	5	Uterus	Blastocysts
		12	12	4	Uterus	Blastocysts
		13	12	1	Uterus	Blastocysts
		14	14	0	—	—
		15	13	0	—	—
			$\bar{x}=12.7$	$\bar{x}=3.2$		
	6	16	15	7	Uterus	Blastocysts
		17	12	6	Uterus	Träger present
		18	11	2	Uterus	Blastocysts
		19	13	0	—	—
			$\bar{x}=12.8$	$\bar{x}=3.7$		—
10 μg ECP	2	20	*	6	Oviduct	
		21	*	18	Oviduct	
	3	22	10	1	Oviduct	2 celled
		23	15	0	—	—
		24	17	0	—	—
		25	13	0	—	—
		26	11	0	—	—
		27†	13	10	Uterus	2–3 celled
		28†	12	7	Uterus	2 celled
		29†	11	10	Uterus	2 celled

* Corpora lutea not counted accurately.
† Uteri ligated at day 1.

ova recovered from the reproductive tract (Table 2). Some animals had a normal complement of tubal ova at 3 days after mating, in others the number of ova was reduced or eggs were completely absent. Normally at this time, all of the ova would usually be found in the oviduct. There was no correlation as to the age or weight of individual animals and number of ova recovered from different rats at Day 3. All of the ova recovered on the 3rd day appeared to be normal 2–4 celled eggs. The mean number of corpora lutea in the 7 animals killed on Day 3 was 11.6, compared to 5.4 ova recovered per animal (Table 2); approximately 50% of the ova were missing.

In animals receiving 5 μg of ECP, 70 to 75% of the ova were missing from the uterus on Days 5 and 6. Some cornua contained roughly 50% of the ova that had been ovulated; in other tracts few if any blastocysts were recovered (Table 2). All of the recovered blastocysts appeared to be normal.

The effects of 10 μg of ECP on ova transport were much more pronounced and uniform than when 5 μg was injected. On Day 2, 2-celled eggs were flushed from the oviducts of two animals (Table 2). In contrast, on Day 3 only 1 egg was recovered from either the oviducts or cornua of 5 rats. In view of the previous experiments on the effects of ECP on motility of the reproductive tract of the rabbit (12), it seemed likely that the hormone was accelerating egg transport into the uterus and the ova were then being expelled into the vagina. To test this possibility, 3 rats were injected with 10 μg of ECP and the cornua were ligated at the cervical end at 1:00 PM on Day 1. When killed at Day 3, 2- and 3-celled ova were recovered from the cornua of all three animals (females 27–29, Table 2).

The effects of ECP on deciduomata formation. Deciduomata formation was not impaired by the injection of 1 or 5 μg of ECP (Table 3). With the exception of 1 animal in the 1 μg group, all of the traumatized cornua weighed

TABLE 3. THE EFFECTS OF ECP ON DECIDUOMATA FORMATION

Treatment	No. animals	No. with positive response	Mean wt. of positive traumatized cornu (±S.E.) (mg.)	Wt. of control cornu (±S.E.) (mg.)	No. of embryos in control cornua
0.1 ml. cottonseed oil	5	5	790 ± 62	315 ± 31	6, 5, 5, 2, 1
1 μg ECP	5	5	748 ± 87	422 ± 39	7, 6, 4, 3, 0
5 μg ECP	5	5	960 ± 130	330 ± 40	7, 4, 1, 1, 0
10 μg ECP	5	2	970 ± 60	257 ± 38	1, 0, 0, 0, 0

more than 600 mg. The range in weight of deciduomata was greater in the 5 μg treated animals than in the control group or those receiving 1 μg as indicated by a comparison of the standard errors (Table 3). After injection of 10 μg ECP a positive deciduomal response was found in only 2 cornua out of 5 but in both cases the reaction was maximal (Table 3). It is noteworthy that the number of embryos in the control horn diminished with increasing dosages of ECP. This may explain the reduced weight of the control uterus as larger amounts of ECP were injected (Table 3).

DISCUSSION

After a single injection of 10 μg ECP on Day 1 the rats had lost 97% of their embryos when examined at 9–11 days *post coitum* (Table 1). The data in Table 2, clearly indicate that most of the embryos were missing by Day 3 due to accelerated transport of the ova. Two-celled eggs were still present in the oviducts on Day 2 but 24 hours later the ova had passed rapidly through the uterus and into the vagina. Rabbits injected with 25 μg of ECP immediately *post coitum*, also had tubal ova at 24 hours p.c. but by 48 hours very few eggs were recovered from the reproductive tract (12).

In the present study, deciduomata formation was blocked in 3 out of 5 rats treated with 10 μg of ECP on Day 1 (Table 2). Apparently, the level of

circulating estrogens was still sufficiently elevated on Day 5 to interfere with the progesterone controlled decidual reaction. In view of the rapid transport of ova in these experiments, the impaired response of the endometrium is of secondary importance in embryonic death.

The results when 5 μg of ECP were injected were not as clear-cut as at the 10 μg dosage level. Embryos were missing from 4 tracts by the 11th day but variable numbers of embryos had successfully implanted in other cornua. The fact that normal implantation sites were found in some animals suggested that the endometrium was unaffected by 5 μg of ECP. This was confirmed by the 4+ decidual response that was elicited in all of the animals injected with 5 μg of ECP (Table 3).

The effects of 5 μg of ECP on ova transport varied considerably from animal to animal (Table 2), but evidence of acceleration and consequent loss of embryos was shown in almost every instance. Only 29% of the ovulated eggs were recovered as blastocysts from the Day 6 uterus compared to the implantation rate of 35% of the embryos at days 9–11 p.c. (cf. Tables 1 and 2). The lower percentage of ova found on Day 6 probably resulted from the failure to recover all of the eggs from the uterus. This may have been due either to faulty technique or to blastocysts that had already begun to attach to the endometrium and could not be washed free.

Apparently, ECP did not exert a direct effect on the ova. This conclusion is based on the normal appearance and rate of cleavage of the tubal eggs and the normal development of the blastocysts. Therefore, embryonic mortality was primarily an indirect result of the effects of ECP on oviducal and uterine motility.

The present observation that estrogens in certain dosages accelerate the rate of tubal passage of ova is not a new one. Burdick and his associates in a series of papers (13–18) reported that large amounts of estrogen accelerated ova transport while small amounts retained ova in the oviduct where the eggs ultimately dengenerated. Contrary to their finding in the mouse, in the rabbit, 25 μg of ECP accelerated and 250 μg ECP delayed ova transport (12).

Tubal-locking of ova has frequently been used to explain the reduction in litter size that occurs after estrogen treatment (5, 9) but in these studies the oviducts were not flushed to ascertain whether ova were still present at 4 to 6 days *post coitum*.

In preliminary experiments (Greenwald, unpublished) it has been extremely difficult to tube-lock ova in rats or hamsters with a single injection of ECP (5–250 μg) or with multiple injections for 3 days of estradiol benzoate (10 μg) or estrone (5 to 10 μg). On the contrary, in almost every animal these treatments hastened ova transport.

Increasing motility of the reproductive tract at a time when post-ovulatory secretion of progesterone usually diminishes activity could be a promising approach to the problem of fertility control. However, in the present

study, 10 μg of ECP prevented deciduomata formation in some animals as well as accelerating motility of the tract. In addition, the large number of extra-genital reactions in which estrogens participate (19) may also preclude their use as anti-fertility agents. It is possible that compounds which accelerate the motility of another smooth muscle system—the digestive tract—may be equally effective in expelling ova from the uterus without interfering with other reproductive processes. It will be interesting in future experiments to determine whether such drugs can be used to interrupt pregnancy in laboratory animals.

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THE PARATHYROIDS AND THE INTESTINAL ABSORPTION OF CALCIUM, STRONTIUM AND PHOSPHATE IONS IN THE RAT[1]

R. H. WASSERMAN AND C. L. COMAR

Department of Physical Biology, New York State Veterinary College, Cornell University, Ithaca, New York

ABSTRACT

The effect of parathyroidectomy (PTX) on the intestinal absorption of calcium, strontium and phosphate ions was investigated *in vivo* and *in vitro* in the rat. At either 5 or 24 hours after parathyroidectomy, the plasma calcium was significantly decreased in comparison to the controls (mock operated) which indicated the success of the operation. It was observed that PTX did not alter the degree of absorption of Ca^{45}, Sr^{85} or P^{32} by rat duodenum and ileum *in vivo*. In rats raised on a high calcium diet, there was an apparent slight decrease in deposition of Ca^{45} and Sr^{85} in the femur; in rats given a low calcium diet for two weeks, PTX did not influence femur deposition of alkaline earths in respect to the controls. *In vitro* studies with isolated everted gut segments indicated that the transfer of Ca^{45} and Sr^{85} across the duodenum and ileum was not influenced by the parathyroid status of the donor rat.

EVIDENCE presented by Talmage and Elliott (1) indicated that parathyroidectomy (PTX) in the rat decreased the degree of intestinal absorption of Ca^{45} and Sr^{85}. *In vitro* studies by Rasmussen (2) showed that everted duodenal segments from PTX rats did not transport calcium to the same extent as those from the controls. In contrast, Gran (3) recently reported that parathyroidectomy in the rat did not alter calcium absorption; it was further shown that calcium absorption was independent of plasma calcium levels and that PTX did not impair the enhancing effect of vitamin D on calcium absorption in the rachitic rat.

Since earlier investigations in this laboratory had suggested that parathormone was without influence on calcium and strontium absorption, additional work was undertaken to examine in further detail the effect of parathyroidectomy on the movement of Ca^{45} and Sr^{85} across the intestinal barrier both *in vivo* and *in vitro*.

METHODS

In vivo—In these studies, Carworth rats weighing approximately 150 gm. were used. The animals were kept on either a low calcium diet (0.2% Ca) or a high calcium diet (2.0% Ca) for at least two weeks prior to operation. The parathyroid glands were re-

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moved under light anaesthesia, leaving the thyroid tissue intact; the controls were subjected to a mock operation. At 5 hours, the animals were given by a gavage 1 ml. of a solution containing 2% calcium as the acetate salt; experience had shown that rats without supportive calcium treatment usually died in tetany at 8–12 hours post-operation. At 24 hours, the rats were again anaesthetized with ether and a laparotomy performed. One ml. of a solution containing 20 μc Ca^{45}, 2 μc Sr^{85}, and 1 mg. of calcium carrier was injected into a ligated segment of either the duodenum or the ileum. At 2 hours post-injection, the rats were killed and the intestinal segment and femur removed for radioassay of Ca^{45} and Sr^{85} by usual procedures (4). The degree of intestinal absorption was estimated by subtracting the residual radioactivity, in percent of administered dose, from 100. The femur data are also given in terms of "percent of administered dose."

In vitro—In this series the animals were handled essentially the same as in the *in vivo* studies, except for one group which was examined at 5 hours after parathyroidectomy. The *in vitro* procedure was similar to that described by Schachter and Rosen (5), as follows: Rats were killed by exsanguination, the duodenum resected at the pylorus distally and the ileum from the ileo-cecal junction proximally. Intestinal segments (5–6 cm. long) were washed, everted and rewashed in cold isotonic saline, tied at one end with suture, injected with 0.6 ml. of incubating solution, and the other end tied. The intestinal sacs were placed into 25-ml. Erlenmeyer flasks, followed by 3 ml. of cold incubating medium. The flasks were gassed 1 minute with O_2, stoppered, and incubated in a shaking water bath with temperature controlled at 37° C for 1 or 2 hrs. The incubating medium was that of Schachter and Rosen (5) and consisted of NaCl, 0.135M; KCl, 0.011M; $CaCl_2$, 4×10^{-5}M; sodium phosphate buffer at pH 7.4, 0.008M. When added, Ca^{45} and Sr^{85} were present in concentrations of 0.04 and 0.02 μc per ml., respectively. At the end of the incubation, the residual volume of the solution within the sac was determined. Radiocalcium (Ca^{45}) was counted by direct plating on stainless steel planchets with a thin-windowed geiger tube (self absorption corrections were unnecessary). Radiostrontium (Sr^{85}) was determined in well-type scintillation detector according to usual procedures. The data are presented primarily as ratio of counts/minute/ml. on the serosal side (inside of sac) to counts/minute/ml. on the mucosal side (outside of sac); this ratio will be abbreviated as S/M after Rasmussen (2). The comparative behavior of alkaline earths is given in terms of ratio of Sr^{85}/Ca^{45} on the serosal side to that of Sr^{85}/Ca^{45} on the mucosal side.

RESULTS

In preliminary studies, it was shown that parathyroidectomy was without effect on the appearance of Ca^{45} and Sr^{85} in rat femur after an oral dose of radionuclides in the rat. In the presently described studies, the influence of parathyroidectomy on the absorption of alkaline earths from the ligated rat duodenum and ileum as well as the accumulation of the radionuclides by the femur over a two-hour period was investigated. The data are presented in Table 1. First, it may be seen that the plasma calcium decreased significantly as a result of parathyroidectomy. At 24 hours after removal of the gland, some of the PTX rats were in the initial stages of tetany in spite of the administration of stable calcium 5 hours after operation; this situation did not appear to influence the results of this study. By further examination of the data, it is apparent that PTX was without significant effect on the duodenal and ileal absorption of Ca^{45} and Sr^{85}. Also, there did not appear to be a significant effect of parathyroid status on the deposition of the

TABLE 1. THE INFLUENCE OF PARATHYROIDECTOMY ON THE ABSORPTION OF Ca^{45} AND Sr^{85} FROM INTESTINAL SEGMENTS *in Vivo**

Group	Treatment	No. per group	Ca content of diet	Plasma Ca	Segment	Ca^{45}		Sr^{85}		Sr/Ca	
						Absorbed	Femur Deposition	Absorbed	Femur deposition	Absorbed	Femur deposition
				(mg.%)		(% dose)		(% dose)			
1	Control	6	0.2%	10.9 ± 0.04	Duodenum	76 ± 2	1.4 ± 0.1	67 ± 7	1.5 ± 0.1	0.88 ± 0.07	1.1 ± 0.03
2	PTX	5	0.2%	7.6 ± 0.05	Duodenum	75 ± 3	1.3 ± 0.1	60 ± 4	1.4 ± 0.1	0.80 ± 0.02	1.1 ± 0.1
3	Control	5	2.0%	9.6 ± 0.05	Duodenum	65 ± 3	1.1 ± 0.1	50 ± 3	0.90 ± 0.05	0.87 ± 0.06	0.77 ± 0.03
4	PTX	7	2.0%	6.5 ± 0.06	Duodenum	63 ± 3	0.7 ± 0.1	48 ± 2	0.72 ± 0.07	0.93 ± 0.03	0.76 ± 0.02
5	Control	6	0.2%	10.0 ± 0.03	Ileum	33 ± 2	0.8 ± 0.1	21 ± 3	0.64 ± 0.07	0.65 ± 0.05	0.80 ± 0.05
6	PTX	5	0.2%	6.4 ± 0.02	Ileum	35 ± 3	0.9 ± 0.2	25 ± 2	0.75 ± 0.17	0.73 ± 0.03	0.79 ± 0.03

* Values represent mean ± standard error of the mean; period of absorption was 2 hr.

TABLE 2. THE INFLUENCE OF PARATHYROIDECTOMY ON THE TRANSIT OF Ca^{45} AND Sr^{85} ACROSS SURVIVING INTESTINAL SEGMENTS *in Vitro**

Group	Treatment	No. per group	Plasma Ca	Incubation time	Site of Radioactivities†	Ca^{45}		Sr^{85}		Sr^{85}/Ca^{45}	
						Duodenum	Ileum	Duodenum	Ileum	Duodenum	Ileum
			(mg.%)	(hr)		(S/M)		(S/M)			
1	Control	6	9.9 ± 0.3	2	Outside (mucosa)	1.9 ± 0.3	0.60 ± 0.02	0.65 ± 0.08	0.39 ± 0.05	0.38 ± 0.05	0.66 ± 0.10
2	PTX	6	6.6 ± 0.5	2	Outside (mucosa)	1.9 ± 0.4	0.59 ± 0.04	0.63 ± 0.05	0.34 ± 0.04	0.40 ± 0.18	0.61 ± 0.07
3	Control	5	11.4 ± 0.3	1	Outside (mucosa)	1.4 ± 0.3	0.41 ± 0.05	0.50 ± 0.03	0.24 ± 0.03	0.40 ± 0.05	0.64 ± 0.13
4	PTX	4	7.3 ± 0.4	1	Outside (mucosa)	1.9 ± 0.5	0.48 ± 0.07	0.42 ± 0.04	0.22 ± 0.01	0.24 ± 0.04	0.51 ± 0.12
5	Control	5	10.2 ± 0.02	2	Inside & Outside	3.6 ± 0.3	—	1.02 ± 0.04	—	0.30 ± 0.02	—
6	PTX	6	5.2 ± 0.03	2	Inside & Outside	3.8 ± 0.5	—	0.94 ± 0.02	—	0.26 ± 0.04	—

* Values represent mean ± standard error of the mean.
† Refers to site of label, i.e., Ca^{45} and Sr^{85} placed either in outside solution only or both inside and outside solutions.

alkaline earths in the femurs of the rats given a low calcium diet for two weeks prior to experimentation. In the series in which the rats were raised on a high calcium diet, there appeared to be a small, but significant, decrease in the deposition of both Ca^{45} and Sr^{85} in the rat femur after parathyroidectomy; yet, the absorption of these ions was unaffected. The alteration in femur deposition could possibly be brought about by secondary effects of parathyroid hormone at the skeletal or kidney level or both.

It may be further seen that calcium absorption from the ileum was significantly less than that from the duodenum; this was expected from earlier observations by Lengemann *et al.* (6) and Cramer and Copp (7). The decreased absorption of calcium by rats raised on a high calcium diet in contrast to those given a low calcium diet reflects an alteration in the efficiency of absorption; this corroborates observations previously made in this and other laboratories (8, 9).

In the *in vitro* study, two types of approaches were employed. In the first series (Groups 1–4), the Ca^{45} and Sr^{85} activity was placed only in the outside solution (mucosal) although the total composition of the bathing fluids was otherwise identical. In this way, the effect of PTX on the movement of the ions by both physico-chemical processes and "active" transport could be observed. In the second series (Groups 5, 6), the radioactivity was present in both the outside (mucosal) and inside (serosal) solutions; here, only an effect of PTX on "active" transport of the ions would be detectable.

From Table 2, it may seem that Ca^{45} was transported against a concentration gradient by the duodenal sac whereas Sr^{85} was not; in the ileum, neither Ca^{45} nor Sr^{85} was transported against a gradient. These observations agree with those of Schachter *et al.* (10) and Wasserman (11). As a result of PTX, the expected decrease in plasma calcium levels was observed. Of primary interest was that the transit of Ca^{45} and Sr^{85} across the duodenal and ileal segments was not influenced by parathyroidectomy; this conclusion holds for the different incubation times and whether the radionuclides were present on both sides or on one side of the membrane. This would mean that neither the transport nor the diffusion of the alkaline earths was influenced by parathormone deficiency under the present *in vitro* conditions.

An additional study was performed in which the influence of parathyroidectomy on the absorption of phosphate-P^{32} from duodenum and ileum was examined (Table 3). It may be seen from the tabulated values that the removal of the parathyroid glands was also without effect on the movement of phosphate across both intestinal segments.

DISCUSSION

Neuman and Neuman (12) and Irving (13), in assessing past informa-

TABLE 3. INFLUENCE OF PARATHYROIDECTOMY ON PHOSPHATE-P^{32} ABSORPTION FROM INTESTINAL SEGMENTS *in Vivo**

Group	Treatment	No. per group	Plasma Ca	P^{32} Absorption	
				Duodenum	Ileum
			(mg. %)		
1	Control	6	9.7 ± 0.02	85 ± 1	40 ± 7
2	PTX	7	7.8 ± 0.03	86 ± 1	55 ± 11

* Values represent mean ± standard error of the mean.

tion, concluded that parathormone has a negligible effect on the absorption of calcium from the intestine. In the present studies, this general concept was verified in that parathyroidectomy did not alter the movement of Ca^{45} and Sr^{85} across the intestine both *in vitro* and *in vivo;* also parathormone was without effect on the absorption of phosphate-P^{32} from the duodenum and ileum *in vivo*. This corroborates the findings of Gran (3) in which a parathyroid response on calcium absorption was not observed in the vitamin D-deficient rat. As mentioned previously, Talmage and Elliott (1) found a clear-cut reduction in Ca^{45} and Sr^{85} absorption *in vivo* after PTX, and Rasmussen (2) also observed a significant effect of PTX on the transport of calcium by excised duodenal sacs *in vitro*. Dowdle *et al.* (14), using everted duodenal sacs *in vitro*, did not observe any alteration in the active transport of calcium in vitamin D-depleted, thyroparathyroidectomized rats; however, after the administration of 50,000 I.U. of calciferol, there was a significantly greater degree of transport of calcium in the control versus the PTX groups.

Since an effect of PTX on the intestinal absorption of calcium cannot be consistently demonstrated, it would seem that any such action of parathormone is small in contrast to the accepted action of the hormone on kidney and skeletal metabolism (15). Also, it certainly appears that the effect of parathormone on the intestine, if it exists, is extremely sensitive to the particular experimental conditions under which the study is carried out.

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EFFECT OF LIMBIC STIMULATION ON RELEASE OF CORTICOSTEROIDS INTO THE ADRENAL VENOUS EFFLUENT OF THE CAT[1]

MARGARET A. SLUSHER AND JANE E. HYDE

Brain Research Institute and the Departments of Anatomy, Physiological Chemistry and Physiology, School of Medicine, University of California Medical Center, Los Angeles, California

ABSTRACT

Facilitatory and inhibitory influences on adrenal cortical secretion have been demonstrated after unilateral stimulation of selected limbic structures in acute encéphale isolé cat preparations. Thus, significant decreases in corticosteroid levels in the adrenal venous effluent were observed in 9 cats, 15 or 30 minutes after a single 30 second stimulation in the uncus, the lateral amygdala, the preoptic region, diagonal band of Broca, or the septum. Significant increases in corticosteroid levels were observed in 4 cats following stimulation in hippocampal cortex or in antero-mesial amygdala. No significant change in corticosteroid levels was seen in 3 cats during a one-hour period following stimulation in other hippocampal or amygdaloid areas. Thus, the limbic system would appear to contain separate components functionally related to inhibition or facilitation of ACTH release.

THE concept of neural regulation of release of ACTH by the adenohypophysis has been based largely on results obtained with stimulation and lesions in the hypothalamus and in other diencephalic as well as mesencephalic areas. Recent investigations in the acute cat preparation (1) extended the functional role of the posterior diencephalon to include an inhibitory in addition to the previously recognized facilitatory influence on regulation of the pituitary-adrenal axis. In addition, an inhibitory influence on ACTH release, as measured by alterations in the level of corticosteroid in the adrenal venous effluent, has been demonstrated following chronic midbrain lesions in the rat (2) and following stimulation in the midbrain tegmentum of the encéphale isolé cat (1).

There have been few earlier attempts utilizing stimulation techniques to relate limbic structures to regulation of ACTH release. Porter (3) found evidence suggesting an inhibitory role of the uncus on the eosinopenic response to injected epinephrine in monkeys. Mason's studies indicated a marked facilitation of ACTH release following 90 minute amydaloid stimu-

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ation in chronic monkey preparations (4). In addition, hippocampal timulation appeared to exert a prolonged suppressive action on the pituiary-adrenal system (5).

In view of the known relationships between emotional behavior and ndocrine output and of the accepted association of limbic structures with motional behavior it was felt of importance to attempt a more systematic tudy of this phase of the regulation of ACTH release, as measured by lterations in adrenal effluent corticosteroid release following 30-second timulation of selected limbic structures in the unanesthetized encéphale solé cat.

MATERIALS AND METHODS

Sixteen adult male cats weighing 2–4 kg. were prepared as encéphale isolés under ether y the method of Bremer (6). Following craniotomy for electrode placement, and section f the spinal cord between C-1 and C-2, ether administration was discontinued and rtificial respiration instituted. A rectal thermometer was inserted and heat lamps adusted for maintenance of body temperature.

Twenty to 30 minutes after cord section, laparotomy and adrenal vein cannulation were performed as described previously (1). Following collection of the first adrenal venous sample, silver ball electrodes were placed on the dura over the right suprasylvian gyrus for electrocorticographic (ECG) recording (Offner type D EEG). A bipolar concentric electrode (No. 26 gauge) was inserted stereotaxically for subsequent unilateral stimulation. The femoral artery and vein were cannulated for manometric recording of arterial blood pressure and for i.v. drip of whole cat blood. The heart rate was recorded as an EKG between a leg clip and one cortical lead. The left adrenal vein was cannulated and adrenal venous samples were periodically collected and prepared as previously described for chromatography and colorimetric analysis of corticosteroid levels (1). Four hours after cord section a 3–5 min. effluent sample was collected and immediately thereafter a 30 second train of square waves was delivered from a Grass S-4-B stimulator, at 25 per second, pulse duration 15 msec., 3.0 volts. Adrenal venous samples (average 5 min. duration) were collected during the subsequent two hours (cf. Table 1).

All cats were monitored for blood pressure, heart rate, body temperature, pupillary size and light reflexes, spontaneous and alerting movements of eyes, and ECG. The experiment was terminated for any animal whose blood pressure dropped to 60 mm. Hg, whose pupillary light reflex was sluggish, who exhibited anisocoria, or whose ECG was abnormal. Terminally, stimulated sites were lesioned (3 mamps D.C., 5 sec.) for subsequent identification and reconstruction. Brains were fixed in formalin, sectioned at 80 μ, and stained with thionin.

RESULTS

Previous studies (1) on six non-stimulated control cats revealed a maximal variation of plus or minus 25% during any two hour period for adrenal corticosteroids collected between 3 and 10 hours after cord section. On this basis, corticosteroid levels in the present study were considered significantly elevated after stimulation if higher than 25% over pre-stimulation levels, and were considered significantly decreased if depressed more than 25%. All percent changes are expressed relative to the immediate prestimulation value for each cat, this value being equivalent to 100%.

TABLE 1. EFFECT OF LIMBIC STRUCTURE STIMULATION ON ADRENAL EFFLUENT CORTICOSTEROID LEVELS

Cat group	Histological location of stimulated site	Corticosteroids, total μg/5 minutes (single samples)					
		Immed. pre-stim.	Minutes post-stimulation				Percent change
			0–5	15–20	30–35	60–65	
A	amygdala (baso-lat.)	13.4	—	*7.8*	8.5	15.0	−42
	amygdala (lat.)	7.9	8.3	6.4	*2.4*	—	−69
	amygdala (lat.)	4.6	5.3	3.8	*2.7*	2.6	−41
	hippocampus (uncus)	11.7	—	*7.2*	10.2	14.7	−38
	hippocampus (uncus)	7.1	5.7	*4.6*	2.7	7.3	−35
	preoptic region	4.2	3.2	4.2	*2.2*	4.3	−48
	diag. band Broca	9.0	—	*4.6*	5.5	7.4	−49
	diag. band Broca	9.0	—	8.0	*4.7*	8.6	−48
	septum-diag. band	6.7	—	*2.6*	4.8	4.5	−61
B	amygdala (baso-med.)	6.0	4.8	*8.2*	7.3	7.8	+37
	amygdala (baso-med.)	5.0	5.6	6.2	*7.8*	3.0	+56
	amygdala (baso-med.)	2.3	—	*4.5*	5.2	3.6	+96
	hippocampus (dentate gyrus)	12.4	*22.1*	17.7	15.9	10.2	+78
C	amygdala (baso-lat. to baso-med.)	4.9	3.4	4.8	5.3	5.8	
	hippocampus (entorhin.)	4.9	—	5.9	6.1	—	
	hippocampus (entorhin.)	3.9	4.6	4.2	3.8	4.6	

Italicized data represent first significant change in 30 minutes from pre-stimulation levels. These values are expressed as percent change.

In 16 cats, sites in the amygdaloid nuclei, or their projection systems (7) were stimulated for 30 seconds, 4 hours after cord section. Periodic levels of adrenal effluent corticosteroids and the first significant percent changes are presented in Table 1.

Significant decreases in adrenal effluent corticosteroid levels, of −35 to −69%, were observed 15 or 30 minutes after 30 second stimulation in 9 cats (Group A). Samples of effluent collected immediately post-stimulation in 4 cats of this group revealed no significant alteration of corticosteroid levels when compared with pre-stimulation values. In 7 of 9 cats exhibiting inhibited corticosteroid release, recovery toward pre-stimulation levels was seen 1 hour after stimulation. Sites, the stimulation of which resulted in decreased corticosteroid release, were located in hippocampal uncus, lateral nucleus or lateral part of basal nucleus of amygdala,[2] pre-optic region, diagonal band of Broca, and septum.

Significant increases in adrenal effluent corticosteroid levels, of +37 to +96%, were observed during the first 30 minutes after stimulation in 4

[2] Gloor terminology is used for amygdaloid nuclei (7). Histological localization of amygdaloid sites was reconstructed using the atlas of Jasper and Ajmone-Marsan (8). Baso-lateral sites were in N. amygdaloideus basalis, pars magnocellularis; lateral sites were in N. amygdaloideus lateralis; basomedial sites were in N. amygdaloideus basalis, pars parvocellularis.

cats (Group B). With stimulation of sites located in the medial part of the basal nucleus of the amygdala, these increases were significant 15 or 30 minutes post-stimulation. After stimulation of a site in the hippocampus in one cat, corticosteroids collected during the first 5 minutes were elevated 78% above the immediate pre-stimulation level. Of the total 9 cats in which samples were collected 0 to 5 minutes post-stimulation, this was the only instance in which an immediate response was noted. The site which elicited this response was histologically located in the anterodorsal aspect of the dentate gyrus. Corticosteroid levels indicated a return toward pre-stimulation levels by 1 hour post-stimulation in 3 of 4 cats exhibiting a facilitated release of corticosteroids.

No significant alteration in adrenal effluent corticosteroid levels was found during the 1 hour period after stimulation in 3 cats with other limbic placements (Group C). Sites tested were located either in the entorhinal cortex or in the amygdaloid complex on the border between the baso-medial and baso-lateral nuclei.

No cortical seizure activity was recorded following either insertion of the stimulating electrode or stimulation at 25 per sec., 15 msec., 3.0 volts in any cat represented in Table 1. Data were discarded from 3 additional cats, in which ECG seizures were noted following electrode insertion into the hippocampus.

DISCUSSION

Under the conditions of the present study, 30 second stimulation of specific limbic sites resulted in a significant increase or decrease in corticosteroid levels in the adrenal venous effluent. Thus, there would appear to be components in the limbic system capable of both facilitating and inhibiting the pituitary-adrenal axis. Under our experimental conditions, these components appear to have a topographical localization with the amygdala and hippocampus.

Other studies attempting to relate limbic structures to ACTH release have employed different techniques and assay methods. The most relevant study with respect to facilitatory components within the limbic system is that of Mason (4). In experiments on a chronic monkey preparation, significant elevation of plasma 17-OH corticosteroid levels was noted following 90 minute stimulation of diverse sites throughout the amygdaloid complex. These data are in contrast to the present observation where an increase or decrease of adrenal effluent corticosteroid levels was found 15 to 30 minutes following stimulation in specific subdivisions of the amygdala. Evidence for components of an inhibitory nature within the amygdala was reported by Martin *et al.* (9), who found significant elevation of adrenal effluent corticosteroid levels following chronic amygdalectomy in cats and dogs.

Stimulation of the uncus of the hippocampus in the present experiments

was noted to result in an inhibition of corticosteroid release (cf. also, Porter, 3).

Stimulation of the rostral mid-line structures of the limbic system revealed only inhibitory effects on corticosteroid release. Previous work (1) had demonstrated only inhibition of corticosteroid release following stimulation in the midbrain tegmentum. No attempt has been made to map either the midline limbic structures or the midbrain with respect to influences on corticosteroid secretion. It is possible that a more extensive survey of each area would reveal facilitatory as well as inhibitory components for ACTH regulation.

In conclusion, the present experiments indicate that ACTH release may be inhibited as well as facilitated following stimulation within the limbic system, but do not explain the mechanism of inhibition of pituitary-adrenal function. Whether an inhibitory stimulus ultimately induces an activation of certain neuronal elements, or acts passively to dampen or prevent the flow of facilitatory impulses which normally result in ACTH release, can only be determined by further experiments.

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NOTES AND COMMENTS

INTERFERENCE WITH DEIODINATION OF SOME THYROXINE ANALOGUES IN THE RAT

ABSTRACT

Triiodothyronine, its propionic acid analogue and D-thyroxine are rapidly deiodinated by the rat. Propylthiouracil greatly reduced the rate of deiodination of these compounds and resulted in higher concentrations of PBI than were found in control animals whose thyroids were blocked by potassium perchlorate. The effectiveness of the thyronine derivatives to prevent goiter was influenced by an extrathyroidal effect of propylthiouracil.

It is possible that the relative effectiveness shown by different thyroxine analogues upon oxygen consumption, cholesterol metabolism, goiter prevention, growth and metamorphosis may be related to the biological half-life of each analogue. One criterion of the biological half-life is the rate of deiodination of the iodinated compound.

In vivo thyroxine metabolism was normal in presence of potassium perchlorate (1) or methimazole[1] (2) but deiodination was greatly reduced by as little as 50 micrograms of propylthiouracil in the rat (1). Other investigators (3, 4) have shown that rat kidney slices will change L-thyroxine to a compound, chromatographically similar to triiodothyronine, and this conversion was prevented by thiouracil derivatives.

This report summarizes studies regarding an effect of propylthiouracil upon the metabolism and effectiveness of triiodothyronine, triiodothyropropionic acid and D-thyroxine in rats. The biological effectiveness was measured by the ability of each compound to prevent goiter in presence of propylthiouracil and of potassium perchlorate.

METHODS

I-131 labeled analogues[2] of thyroxine were prepared with low specific activity in a 0.02N NaOH solution. Each day a constant volume of the solution was injected subcutaneously into adult male rats, as described previously (1). Thyroid utilization of radioiodine was prevented by 1% potassium perchlorate in drinking water or by the addition of 0.03% propylthiouracil in the diet. The animals were fed a low residue, low iodide synthetic diet (1). Urine and feces were collected every two days. In studies with radiothyroxine (1), equilibrium of the plasma PBI-131 and PBI-127 was 95% complete within 10 days. Equilibration was assumed for the other compounds when I-131 balance was attained. Paper chromatography (1) showed only iodide in the urine of rats given 3,5,3′ triiodothyropropionic acid[3], 3,5,3′—triiodo-L-thyronine[4] and L-thyroxine[5]; urinary I-131 from D-thyroxine[5] was assumed to be iodide. Frequent chromatography showed the injection solutions always contained more than 86% of the I-131 in the organic compound.

RESULTS

Table 1 lists the daily urinary excretion of I-131 on the tenth day of the experiment, after urinary I-131 had been constant for 4 to 6 days. Rats fed potassium perchlorate were considered to be controls (1) and they rapidly excreted radioiodide from the labeled compounds. Propylthiouracil reduced the deiodination of each compound, the absolute reduction was 1.0 to 1.3 μg/day from the 2.5 μg daily dose of L-thyroxine, D-thyroxine or triiodothyropropionic acid. The triiodothyronine doses were much smaller and cannot

[1] 1-Methyl-2-mercaptoimidazole.

[2] Abbott Laboratories, Oak Ridge, Tennessee.

[3] Warner-Chilcott Laboratories, Morris Plains, New Jersey.

[4] Smith Kline and French Laboratories, Philadelphia, Pennsylvania.

[5] Travenol Laboratories, Morton Grove, Illinois.

TABLE 1. URINARY I-131 AND GOITER PREVENTION FROM THYROXINE ANALOGUES INJECTED DAILY INTO RATS FED $KClO_4$ OR PROPYLTHIOURACIL
(8 rats for each dose)

		Average body wt. initial/final, gm.		Daily urinary I-131 (% of I-131 in daily dose)		Thyroid size, mg./100 gm. final body wt.		Final PBI (calculated* μg %)	
Antithyroid compound →		$KClO_4$	PTU	$KClO_4$	PTU†	$KClO_4$	PTU	$KClO_4$	PTU
I-131 Compound	Dose μg rat/day								
Triiodothyronine	0.3	154/198	149/189	90	51	16	16	0.04	0.09
	0.6	123/165	123/145	—	—	5.7	6.8	—	—
Triiodothyropropionic acid	2.5	146/183	151/197	90	39	15	15	0.21	0.87
	5.0	142/187	140/173	86	41	11	7	0.38	1.47
D-thyroxine	2.5	186/226	188/242	86	41	23	29	1.8	5.2
L-thyroxine	1.4	136/218	132/220	68	29	9	18	2.8	5.2
	2.5	137/232	134/202	70	28	5	8	6.8	9.9
Average Control	—	134/220		—		9.0		3.0	

* 24 hours after last dose of Iodothyronine, see reference (1) for calculation of μg PBI from I-131 measurements.
† 6-n-propyl-2-thiouracil.

be compared quantitatively with the other compounds. In all comparable experiments the PBI-131 was greater in rats given propylthiouracil than those fed potassium perchlorate.

Triiodothyropropionic acid was readily deiodinated and rapidly disappeared from the plasma. Control rats injected only once with I-131 triiodothyropropionic acid (Table 2) cleared their plasma of PBI-131 two to four times faster than similar animals fed propylthiouracil. Two hours after the injection of controls, 53% of the plasma I-131 was PBI-131; this compared to 91% of plasma radioiodine that was protein-bound in rats fed propylthiouracil.

The data of Table 1 show the goiter prevention dose of triiodothyronine was similar in presence of either antithyroid compound. In contrast to L-thyroxine (1), 5 μg per day of triiodothyropropionic acid prevented goiter more effectively in presence of propylthiouracil than in presence of potassium perchlorate ($P = <0.01$). Histology of the thyroid of rats given triiodothyropropionic acid in the presence of potassium perchlorate showed severe hyperplasia. In the presence of propylthiouracil the plasma concentration of triiodothyropropionic acid remained elevated with the result that macroscopic and microscopic hyperplasia of the thyroid was prevented.

DISCUSSION

Propylthiouracil greatly reduced the deiodination of the thyroxine analogues included in this study. Such action of propylthiouracil could reduce the effectiveness of

TABLE 2. PLASMA PBI-131 IN RATS AFTER A SINGLE SUBCUTANEOUS INJECTION OF I-131 TRIIODOTHYROPROPIONIC ACID (4 rats had 1% $KClO_4$ in drinking water and 4 had 0.03% PTU in food)

Hours after injection	PBI-131 (% of dose/cc.)	
	$KClO_4$	Propylthiouracil
1	0.41	0.95
2	0.40	0.98
3	0.31	0.83
6	0.13	0.52
12	0.07	0.32
24	0.05	0.18

thyroxine, if deiodination were to result in the production of a more effective compound or if deiodination were essential for the biological effectiveness. This effect of propylthiouracil (6, 7) might explain the fact that PBI in rats is abnormally elevated when propylthiouracil goiter is prevented by thyroxine injection (8, and Table 1). A similar mechanism was proposed by Wilkinson *et al.* (5), who regarded the antithyroxine activity of n-butyl 4-hydroxy-3:5-diiodobenzoate as caused by interference with the production of triiodothyronine from thyroxine.

The biological importance of deiodination is unknown, and it is possible that part of the deiodination could prevent some thyroxine analogues from exhibiting their thyroxine-like effect. In that case, propylthiouracil should preserve the compound and might result in increased biological effectiveness. Triiodothyropropionic acid was the only compound that we found to prevent goiter at a smaller dose with propylthiouracil than with perchlorate. However, it is misleading to compare biological effectiveness only on the size of the administered dose of the compound when the concentration in the plasma depends upon more than the dose.

Finally, deiodination may both potentiate and "waste" thyroxine analogues and the portion which goes by each route may be decisive in determining the biological effectiveness. Such a complex system might be partially resolved if we had more information regarding the mechanism of the extra-thyroidal action of propylthiouracil. It is curious that propylthiouracil prevents iodination in the thyroid gland and inhibits deiodination in the extra-thyroidal tissues.

Acknowledgment

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L. Van Middlesworth and S. L. Jones

Department of Physiology
University of Tennessee
Memphis, Tennessee

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THE STAIRCASE PHENOMENON IN THE MYOMETRIUM OF THE RAT

ABSTRACT[1]

Three types of staircase phenomena were observed *in vitro* in the pregnant and *post partum* rat myometrium by varying the frequency of electrical stimulation. A positive staircase (positive correlation between frequency of stimulation and amount of tension developed) was occasionally observed on day 1 of pregnancy. This response rapidly changed to a transient and then to a negative staircase within a few hours. The negative staircase was observed throughout the remainder of pregnancy and for the first few hours following parturition. At approximately 8–12 hours *post partum* the predominant type of staircase was of the transient type which changed to the positive type at about 24 hours *post partum*. A few positive staircase responses were recorded on day 19 of pregnancy, i.e. as early as three days prior to parturition. Uteri of ovariectomized rats treated with estrogen, or estrogen and progesterone, showed only the negative type of staircase.

While determining the optimum frequency of electrical stimulation for development of maximal tension in the rabbit myometrium, Csapo and Corner (1) found the estrogen-dominated uterus to exhibit a positive staircase similar to that observed for the frog's heart (Bowditch, 2; Hajdu, 3). This may be described as an increase in the amount of tension developed by the myometrium as the frequency of stimulation is increased. However, the progesterone-dominated uterus showed a decrease in tension with increasing frequency of stimulation (negative staircase). Subsequently, Schofield (4, 5) obtained similar results in both the pregnant and ovariectomized, hormone-treated rabbit when tension was recorded *in vivo*. Moreover, observations in the pregnant rabbit (5) and ewe (6) showed a change in hormonal domination of the myometrium from progesterone to estrogen at the end of pregnancy.

Recently, Schofield (7), using both *in vivo* and *in vitro* recording procedures, reported that only a negative staircase effect was observed with the rat myometrium over the last 8 days of pregnancy, during parturition and immediately *post partum*. In addition, a positive staircase was never obtained in castrated, estrogen-treated rats (injected daily with doses up to 100 μg for 4–12 days). The present study is concerned with the staircase phenomenon in the rat myometrium and reports the presence of positive, transient and negative staircases under various physiological conditions.

MATERIALS AND METHODS

Uteri employed in this study were obtained from albino female rats (Purdue strain) under well defined conditions of treatment or physiological state. In all experiments performed on uteri of castrated, hormone-treated rats, animals weighing 200–250 g. were ovariectomized under ether anesthesia. At least three weeks were allowed for uterine atrophy after which time all dosages of estrogen (estradiol benzoate or estradiol cyclopentylpropionate)[2] and progesterone were injected intramuscularly in 0.1 ml. peanut oil. Approximately 24 hours following the last injection of hormone the animals were decapitated, the uterus rapidly removed in placed in Krebs solution. If the uterus was not to be used immediately it was stored in Krebs solution at 4° C for use later the same day.

Experiments performed on uteri of pregnant rats were carried out in the following manner. Male rats were placed with the female rats at 9 P.M. At eight o'clock the following morning, day 1 of pregnancy was determined by the appearance of sperm in the vaginal smear. The uteri were removed at stated intervals as indicated above. Following removal of the uterus from the animal, the fetuses were expelled by gently loosening the placenta and allowing the conceptus mass to slide out of one end of the uterine horn. The uterus was then quickly placed in Krebs solution. Removal of either a gravid or non-gravid uterus could be completed within 1–2 minutes following decapitation.

The apparatus used for measurement of uterine tension consisted of the following components: (1) a 50 ml. muscle chamber mounted in a constant-temperature water bath, (2) an isometric muscle level connected by a fine chain to a transducer (Stratham, Model G7A-0.15-350), and (3) a Sanborn Twin-Viso recorder. One uterine horn, or a segment of muscle having 4–5 implantation sites when a pregnant uterus was employed, was suspended

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[2] Aided in part by a grant (RG-7831) from the NIH.

in a vertical position by attaching it with surgical thread to a glass hook at the bottom of the chamber and to the isometric tension lever at the top of the chamber. The Krebs solution in the muscle chamber contained (mM/liter): NaCl, 118.46; $NaHCO_3$, 24.87; KCl, 4.74; $CaCl_2$, 2.54; KH_2PO_4, 1.18; $MgSO_4$, 1.18; glucose, 5.56. The pH of this solution was 7.4 when it was equilibrated with 95% O_2 and 5% CO_2 before and during the experiment by a continuous flow of gas. A hydrostatic pressure system was used to fill the muscle chamber rapidly with Krebs solution, which was heated to 37° C by allowing the solution to flow through glass coils in the water bath. Rapid emptying of the chamber was accomplished with an aspirator attached to the bottom of the chamber.

Following placement of the uterus in the above apparatus its "resting length" (maximum length of the uterine segment at which it develops no resting tension) was determined by increasing the length of the uterine segment in a stepwise manner until resting tension was barely recorded and then decreasing the length just slightly. The latter was accomplished by means of an isometric tension clamp to which a piece of 8 mm. glass tubing was attached which went down into the muscle chamber along one side. This tube had a glass hook to which the muscle was tied at the bottom. Thus, by lowering the tube with the muscle attached to it the length of the uterine segment was increased. The glass tube also served as the inlet for the gas mixture. The importance of carrying out this operation in each experiment is revealed in the length-tension studies of Csapo and Goodall (8) who demonstrated that maximal tension of uterine muscle is recorded only at its resting length.

The transducer employed was extremely sensitive (an isometric tension level had to be inserted between the muscle and transducer to prevent the latter from being deflected to its maximum with each uterine contraction) and therefore, any minute movement of the isometric tension level, and in turn of the transucer was amplified and recorded as tension. The system was practically isometric in that shortening of the uterine segment was less than 1% of its resting length as determined by a cathetometer. The relationship between the tension recorded and the load applied to the isometric tension lever was linear.

The uterus was stimulated electrically by suspending it between two platinum wire ring electrodes (ca. 20 mm. diam.) which could be adjusted so as to include the entire uterine segment in the longitudinal field. The electric field set up in the Krebs solution between the electrodes consisted of 60 cycle A.C. applied for a duration of 5 sec. with frequencies of one stimulus (tetanus) every 15, 30, 60, 90, 120 and 240 sec. The voltage used corresponded to that of the first peak of tension (Callantine *et al.* 9) on the strength of stimulus-tension curve and was usually about 2 v/cm longitudinal field.

RESULTS AND DISCUSSION

The types of staircase observed in the rat myometrium under various hormonal conditions are shown in Figure 1 and Table 1. All the uteri obtained from ovariectomized hormone-treated rats, whether treated with estrogen alone, or estrogen in combination with progesterone, exhibited a negative staircase. In addition, only negative staircase responses were recorded during the first and second trimesters of pregnancy with the exception of an occasional positive staircase recorded early on Day 1 of gestation. All three types of staircase effects were seen at parturition although the negative staircase was predominant. At 8 to 12 hours *post partum*, the relative number of negative staircase responses decreased and the transient responses increased; while at 24 hours *post partum* the majority of the uteri showed a positive staircase. However, it should be noted that an occasional positive staircase was recorded on Day 19 of gestation, i.e., as early as three days prior to parturition.

Schofield (7) has stated that the negative staircase observed prior to, during, and immediately following parturition was probably not indicative of a different type of hormonal regulation at the end of gestation in the rat as compared to the rabbit and ewe. Rather it was suggested that the myometrium of the rat may have a different organization than that of the other two species, and hence, responds differently to electrical stimulation. However, the present findings indicate that the rat myometrium is also responsive to changes in the ratio of the ovarian hormones since the staircase response was occasionally positive on Day 1 of pregnancy, negative during gestation and positive some hours after parturition. The fact that a positive staircase has not been demonstrated in the uterus of the ovariectomized estrogen-treated rat may mean that the positive staircase in this species depends on the presence of a certain amount of progesterone in addition to estrogen. The presence of a positive staircase at a time when the concentration of progesterone is believed to be low but not absent, i.e., early

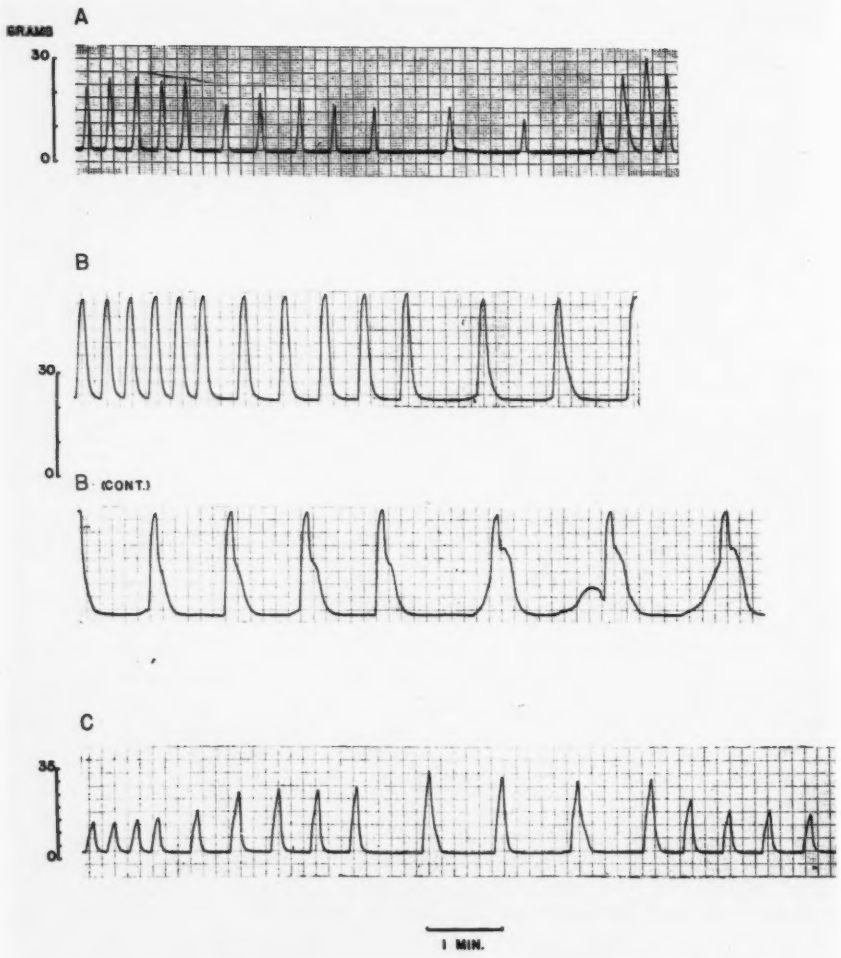

FIG. 1. Three types of staircase phenomena observed in the pregnant rat myometrium by varying the frequency of electrical stimulation. Record A shows a positive staircase, Record B a transient staircase, and Record C a negative staircase.

on Day 1 of pregnancy and 24 hours *post partum*. is evidence for the need of a small amount of progesterone. However, the ratio of estrogen to progesterone required to produce a positive staircase may be rather precise since uteri of ovariectomized estrogen-progesterone treated rats failed to exhibit positive staircases.

Another possible explanation for the failure to obtain a positive staircase following estrogen treatment, or with any consistency at parturition, may be the high degree of spontaneous membrane activity present in the rat myometrium. This could easily interfere with the demonstration of a positive staircase and might explain the positive type of staircase at times when a small amount of progesterone is present in addition to

TABLE 1. STAIRCASE PHENOMENA EXHIBITED BY RAT UTERI UNDER VARIOUS HORMONAL CONDITIONS

No. of animals	Treatment	Staircase
	A. *Ovariectomized Hormone-Treated Animals*[1]	
5	EB[2]—25 μg daily—7 days	negative
5	EB — 4 μg daily—7 days	negative
5	EB — 4 μg daily—7 days Progesterone—1 mg. daily—days 4 to 7	negative
5	ECP[3]—10 μg—day 1	negative
4	ECP —10 μg—day 1 Progesterone—1 mg.—day 7	negative
	B. *Pregnant Animals*	
17	Day 1 of pregnancy	4 positive 13 negative
4	Day 10 of pregnancy	negative
6	Day 15 of pregnancy	negative
15	parturition	2 positive 4 transient 9 negative
11	8–12 hrs. post partum	2 positive 6 transient 3 negative
10	24 hrs. post partum	3 transient 7 positive

[1] All animals killed on day 8.
[2] Estradiol benzoate.
[3] Estradiol cyclopentylpropionate.

estrogen since progesterone raises the membrane potential, and hence inhibits spontaneous membrane activity (9, 10). A high degree of spontaneous membrane activity would tend to lower the threshold and thus make it difficult to record a positive staircase. This possibility must be considered both for uteri obtained from ovariectomized estrogen-treated rats and parturient rats. In the latter case, Marshall (10) demonstrated that oxytocin will cause depolarization and a discharge of action potentials.

MERRITT R. CALLANTINE[3] AND M. X. ZARROW[3]

Dept. of Biological Sciences
Purdue University
Lafayette, Indiana

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EFFECT OF EXPERIMENTALLY INDUCED HYPO- AND HYPERTHYROID STATES UPON PITUITARY LACTOGENIC HORMONE CONCENTRATION IN RATS[1]

ABSTRACT

The lactogenic hormone concentration in the pituitary glands of intact and ovariectomized rats was estimated by the pigeon intradermal method of assay. Administration of Tapazole for 2 weeks caused a large reduction in lactogenic hormone concentration. Reversal of the Tapazole-induced hypothyroid state, i.e. prevention of goitre, with euthyroid doses of thyroxine increased lactogenic hormone concentration considerably above untreated control levels. Injection of hyperthyroid levels of thyroxine caused no apparent change in lactogenic hormone concentration. The same pattern of results was obtained in both intact and ovariectomized groups. It is suggested that the metabolic processes operative in lactogenic hormone synthesis are acutely sensitive to the circulating level of thyroxine.

Numerous studies have shown that, under suitable conditions, the administration of thyroid hormones generally stimulates milk secretion (1). So far, the mechanism of this galactopoetic action has not been elucidated. It has been postulated that thyroxine exerts its effect upon milk secretion by stimulating circulation, thereby providing a richer source of milk precursors (2), by stimulating all the endocrine glands to increased activity (3) or by primarily stimulating increased secretion of lactogenic hormone from the adenohypophysis (4). We have directed our attention to the latter hypothesis in the present study and have investigated the influence of hypo-and hyperthyroid states upon pituitary lactogenic hormone concentration in the rat.

MATERIALS AND METHODS

Intact and bilaterally ovariectomized mature female rats of the Sprague Dawly-Rolfsmeyer strain were maintained at 74–78° F and supplied with Purina lab chow and water *ad libitum*. Castrate rats were injected subcutaneously daily for 2 weeks with 2.5 mg./100 gm. methimazole (Tapazole) alone or in combination with 0.75 or 2.0 μg/100. gm *l*-thyroxine. Intact rats were injected similarly with Tapazole alone or in combination with 1.5 or 2.5 μg/100 gm. *l*-thyroxine. Each rat was killed 24 hours after the last injection and her pituitary and thyroid glands removed and weighed to the nearest 0.1 mg. on a Roller-Smith Torsion balance. Each pituitary gland was frozen. Pituitary lactogenic hormone was analyzed using a modification (5) of the Reese-Turner common pigeon intradermal method of bioassay (6). Four or five pituitary glands were pooled for each assay. These were allowed to thaw at room temperature, crushed in an agate mortar and suspended in distilled water. The suspension was injected over one crop-sac and one of 5 doses of standard lactogenic hormone[2] injected over the other. The amount of lactogenic hormone (X) in each unknown was calculated from measured crop-sac diameters (Y) by the regression equation $Y = 1.92 + 1.33 (\log X)$. The regression equation resulted from the crop-sac responses to various doses of the standard (Fig. 1).

RESULTS

The alteration in thyroid weight and pituitary lactogenic hormone produced by administration of Tapazole alone and combination with thyroxine are depicted in Figure 2. Tapazole produced significant enlargement of thyroid glands and lowered lactogenic hormone concentration in the pituitary from control level in both castrate and intact female rats. Daily injections of 1.5 μg/100 gm. *l*-thyroxine, an amount slightly in excess of the average normal thyroxine secretion rate (euthyroid dose) prevented thyroid enlargement in Tapazole-treated intact rats and increased lactogen/mg. pituitary above untreated control level. Injection of 2.5 μg/100gm. *l*-thyroxine, a quantity in excess of the highest normal thyroid secretion rate observed in this strain (hyperthyroid dose) resulted in pituitary hormone levels essentially the same as untreated controls. Thyroid

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[2] NIH product containing 15 I.U./mg.

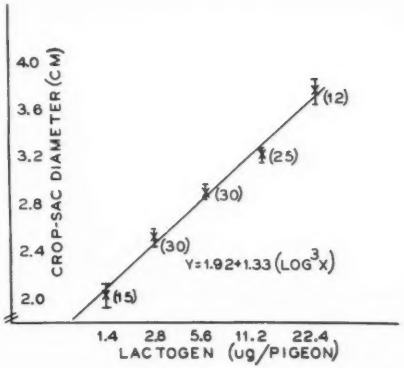

FIG. 1. Crop-sac response of the common pigeon to NIH standard lactogenic hormone (15 I.U./mg). Numbers in parenthesis refer to number of pigeons; vertical bars represent standard error of the mean.

gland weight was reduced still further but remained in the control range. The same pattern of results occurred with euthyroid and hyperthyroid levels of thyroxine in ovariectomized rats. Less thyroxine was administered, however, since it has been shown that the thyroid secretion rate in rats is reduced following ovariectomy (7).

DISCUSSION

Few investigators have explored the idea that the effects of thyroid hormone upon milk secretion might result in part from altered secretion of lactogenic hormone by the pituitary gland. The possibility that less lactogenic hormone is manufactured during hypothyroid states during which time milk secretion is depressed (8), is suggested by the reduction in pituitary concentration of the hormone which follows thyroidectomy (9) or administration of either thiouracil (10) or, in the present study, of Tapazole. Similar treatments also decrease the size and number of the pituitary acidophils (11–14) which are thought to secrete the lactogenic hormone. Any effects upon lactogenic hormone manufacture during euthyroid or hyperthyroid states are less well visualized. It was noted in previous studies that lactogenic hormone content of the female mouse pituitary

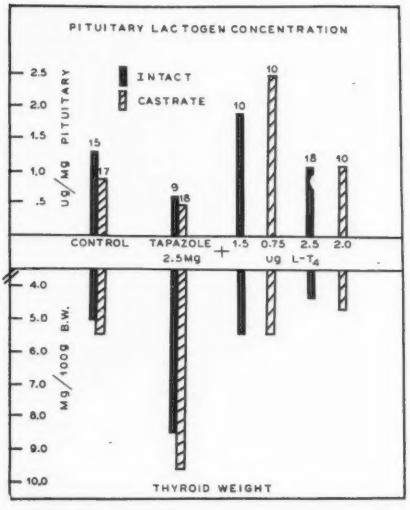

FIG. 2. Experimental alteration of thyroid weight and pituitary lactogenic hormone concentration in female rats. All doses are per 100 gm. body weight; numbers over the bars refer to number of rats.

was unaltered following treatment with large doses of thyroxine (15) while injections of increasingly large doses of thyroxine to male rats decreased lactogenic hormone content of the pituitary (16). Reversal of the Tapazole-induced hypothyroid state in the present study by administration of *l*-thyroxine slightly in excess of the average amounts calculated as normally secreted by intact and castrate rats of this strain (euthyroid dose) not only reversed but increased the pituitary lactogenic hormone concentration considerably above their respective control levels. Injection of hyperthyroid levels of thyroxine (excess of the highest thyroid secretion rates observed in this strain) resulted in pituitary concentration of lactogenic hormone essentially identical with controls.

It is difficult, of course, to interpret a pituitary concentration of lactogenic hormone in terms of its secretion, storage and discharge or to translate pituitary concentration in terms of the milk secretion process. It seems probable, however, that metabolic processes responsible for synthesis of lactogenic hormone are acutely sensitive to the circulating level of thyroid hormone. With the assumption that lactogenic hormone can be galactopoetic (1, 8) in addition to being essential for initiation and maintenance of milk secretion, it is possible that the generally favorable effects of supplemental thyroxine upon milk secretion may be due partly to increased availability of lactogenic hormone for synthesis of milk. Conversely, the depression in milk yield which occurs following administration of large amounts of thyroactive substances (17) may be due partly to reduced lactogenic hormone availability.

CLARK E. GROSVENOR

Department of Physiology
University of Tennessee Medical School
Memphis, Tennessee

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IN VITRO METABOLISM OF STEROID KETALS

ABSTRACT

The effects of 3 or 20 ethylene ketal substituents on adrenocorticoid metabolism have been studied *in vitro*. Ketalization of the 3 position of 9α fluorocortisone significantly reduced metabolism by liver enzymes. Introduction of the ethylene ketal grouping at the C-20 position enhanced steroid disappearance as measured by recovery of isonicotinic acid hydrazide reactive steroid.

Numerous biochemical transformations of adrenocorticoids have been observed using rat liver preparations. Hydrocortisone is reduced by rat liver enzyme systems to the corresponding dihydro and tetrahydro derivatives (1). Moreover, the 20 carbonyl steroid with intact A ring is further metabolized to the pentol (2). *In vitro* studies have established that 1, 2 dehydrogenation inhibits reduction of ring A (3, 4), and a fluorine atom in the 9α-position retards both ring A and C-20 reduction or side chain cleavage (3, 4, 5). Florini and Buyske (5) have shown that 16α-hydroxylation decreases the rate of steroid disappearance.

EXPERIMENTAL

With minor variations, the incubation and extraction techniques of Glenn *et al.* (3) were followed in these experiments. In each experiment control flasks containing only incubation medium and steroid were processed similarly. The residues from 10 ml. aliquots of chloroform-ethyl acetate extracts of the incubation mixture were dissolved in 0.3 ml. of absolute methanol and 0.1 ml. applied across the origin line of 1.5 cm. width prewashed Whatman No. 3 chromatography paper and equilibrated for approximately 4 hours with a Bush system (12 toluene:8 petroleum ether:13 methanol:7 water). Development of chromatogram was conducted for 2 hours. In later experiments the width of the paper was increased to 2.5 cm. and the development time reduced to 1 hour to reduce steroid losses due to overloading and streaking. Authentic steroids consisting of the original ethanolic solutions were run concurrently on control strips. Strips were air dried and all steroids located with the aid of a Mineralite hand lamp (253.7 mμ), with the exception of 5-pregnene-3,11,20-trione-9α-fluoro-17,21-dihydroxy cyclic-3-ethylene ketal. Chromatographic identification of this steroid necessitated running two steroid control strips, one of which was sprayed with blue tetrazolium for location of the parent steroid. Areas corresponding to the authentic steroids were cut out, eluted twice with a total of 10 ml. of methanol and evaporated to dryness. The remaining portions of the strips were carefully examined under ultraviolet light and then sprayed with blue tetrazolium reagent. In no instance was a spot exposed by these techniques. Quantitative analyses were performed by adding the following reagents to the steroid residues:

Blue tetrazolium (*BT*): 2.0 ml. of blue tetrazolium (0.25 mg./ml. absolute ethanol) and 4.0 ml. of 0.003 M alcoholic sodium hydroxide, read at 525 mμ after 1 hour development at room temperature (6).

Isonicotinic acid hydrazide (*INH*): 5.0 ml. of 0.4% alcoholic isonicotinic acid hydrazide (7) and read at 410 mμ after 20 minutes color development at 60° C.

The percent steroid disappearance was calculated by the following formula:

$$\left[100 - \left(\frac{(\mu g.\ \text{steroid recovered from liver incubation})}{(\mu g.\ \text{steroid recovered from control incubation})} \times 100\right)\right]$$

RESULTS AND DISCUSSION

Disappearance of the Δ^4-3 ketone function ($65 \pm 10\%$) of 9α fluorocortisone (Table 1) appeared to be greater than that of the C-20 ketone ($49 \pm 14\%$); however, analysis of these figures indicated that they were not significantly different ($P > 0.3$). Though Glenn *et al.* (3) reported that the rate of C-20 ketone reduction was faster than the rate of Δ^4-3 ketone disappearance, they also indicated that this process could be reversed by alterations in co-substrate and co-factor composition of the incubation medium. The incubation medium of Glenn *et al.* (3) contained triphosphopyridine nucleotide, however, lacked diphosphopyridine nucleotide (DPN). In the present experiments, the

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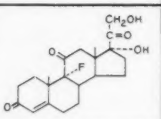
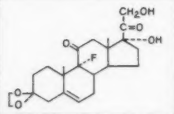
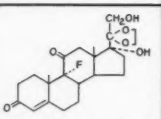
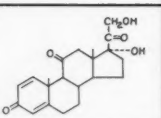
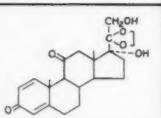
TABLE 1. METABOLISM OF STEROID 3 OR 20 ETHYLENE KETALS

Steroid	Structure	No of Determinations	Color Reagents	R_f	Per Cent Disappearance (Mean ± S.E.)
4-Pregnene-3,11,20-trione-9α-fluoro-17α,21-dihydroxy (9α fluorocortisone)		14 9	BT INH	0.34	49 ± 14 65 ± 10
5-Pregnene-3,11,20-trione-9α-fluoro-17α,21-dihydroxy cyclic-3-ethylene ketal		20	BT	0.71	16 ± 5
4-Pregnene-3,11,20-trione-9α-fluoro-17α,21-dihydroxy cyclic-20-ethylene ketal		11	INH	0.55	93 ± 4
1,4-Pregnadiene-3,11,20-trione-17α,21-dihydroxy (Δ'-cortisone)		8	INH	0.20	56 ± 9
1,4-Pregnadiene-3,11,20-trione-17α,21-dihydroxy cyclic-20-ethylene ketal (Δ'-cortisone-20 ketal)		10	INH	0.41	78 ± 6

BT = blue tetrazolium INH = isonicotinic acid hydrazide

medium also was fortified with 0.01 M DPN. Ketalization of the 3 position of 9α fluorocortisone significantly lessened the ability of liver enzymes to reduce the C-20 ketone to the 20-hydroxy derivative. Reduction of the C-20 ketone is only presumptive since cleavage of the α-ketol side chain also would result in a loss of blue tetrazolium reactive steroid. Introduction of the ethylene ketal grouping at the C-20 position enhanced steroid disappearance as measured by recovery of INH reactive steroid.

It is apparent that the ethylene ketal function can alter the degree of metabolism of 9α fluorocortisone, either increasing or decreasing it, depending upon the position of the ketal moiety.

To further investigate the relation of the 20 ketal configuration to steroid disappearance, Δ'-cortisone-20-ketal and Δ'-cortisone were studied. The results of these experiments substantiated the aforementioned observation that ketalization of the 20 position resulted in a significant increase in steroid metabolism.

Reduction of the 11-keto position of the cortisone analogues also could occur in these incubation studies. As mentioned in the "Experimental" section no BT or ultraviolet positive areas were noted on paper chromatograms, other than for the steroid added to the incubation medium. Thus, if 11-keto reduction did proceed, these metabolites were further degraded.

I. RINGLER AND J. PERRINE[1]

Department of Metabolic Chemotherapy
Experimental Therapeutics Research
Lederle Laboratories
Pearl River, New York

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FORMAZAN FORMATION AS A MEASURE OF HORMONE INFLUENCE ON THE METABOLIC ACTIVITY OF RAT PROSTATE[1]

ABSTRACT

The *in vitro* reduction of triphenyltetrazolium chloride by slices of rat prostate has been used as a measure of metabolic activity of the gland. Marked decreases in formazan production were found upon castration and the levels of reducing systems were elevated upon subcutaneous administration of testosterone or the oral administration of halotestin. Adrenalectomy caused a significant increase in the levels of prostatic reducing systems while cortisol given to intact rats had little effect.

In vitro and *in vivo* formazan production by the reducing systems of tissues has yielded a relatively simple technique for estimating metabolic activity of cells. The ability of various tissues to reduce tetrazolium salts has been utilized in studies on cancer (1), vitamin deficiency (2) and for the assay of hormones (3). That tetrazolium reduction was on estimate of dehydrogenase titers has been demonstrated by increased formazan production in the presence of specific exogenous substrates like succinate. The possibility of employing formazan formation as a measure of metabolic activity of endocrine target organs has been of considerable interest. The present study was undertaken to relate the weight changes of target organs in bioassay procedures to metabolic activity of the tissues. The influence of hormones on rat prostate was evaluated by following both the weight changes of the gland and the ability of the tissue to reduce 2, 3, 5-triphenyltetrazolium chloride.

EXPERIMENTAL

The selection, preparation and particular bioassay run on these animals followed the protocols of Cancer Chemotherapy National Service Center (4). Intact animals weighed between 90–150, adrenalectomized (adrenals removed 5 to 8 days before use) between 60–160 and castrated rats (testes removed the day of assay commencement) 60–145 g.

The incubation procedure and determination of formazan was as previously published (2). Briefly, 60 to 150 mg. wet weight of randomized prostate slices from 8 to 20 rats were incubated in Krebs-Ringer phosphate buffer pH 7.4 containing 0.5% triphenyltetrazolium chloride (TPTZ) The incubation was carried out for 1½ hours at 38° C under air and after one washing of the tissue with 1 cc. distilled water, the red formazan was extracted with 5 to 10 ml. acetone on a mechanical shaker for 20 min. A 2 ml. aliquot of the acetone extract was used for measuring optical density at 480 mμ. A calibration curve of hydrosulfite reduced TPTZ was employed for calculating micrograms of formazan.

Where tissue size was limiting (e.g. untreated castrate rats), only duplicate samples could be run. Sufficient tissue was available from intact and hormone-treated groups to permit quadruplicate determinations.

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RESULTS AND DISCUSSION

The data from these preliminary investigations are outlined in Table I. Formazan production for the intact animals ranged from 97 to 160 μg/100 mg. wet wt. while that for the untreated-castrate groups declined to between 33 to 73. The striking elevation in formazan formation was faithfully reproduced by each group receiving testosterone. The titers for subcutaneously treated castrates ranged from 100–163 when 0.6 mg. testosterone was given and 140–290 when 2.4 mg. testosterone was injected.

Although a rise in TPTZ deposition occurred in halotestine-treated rats, these were of smaller magnitude. The range for 0.6 mg. halotestin was 36–100 and for 9.6 mg. halotestin was 95–204 μg/100 mg. wet wt. whereas the control groups exhibited levels between 19–45.

A noteworthy increase in activity of prostate reducing systems was observed in the adrenalectomized rats. Here formazan levels ranged from 137 to 185/100 mg. wet wt.

TABLE I. THE INFLUENCE OF HORMONES ON THE LEVELS OF REDUCING SYSTEMS IN RAT PROSTATE

Condition of animals	Treatment	No. of animals	No. of studies*	Formazan μg 100 mg. wet wt.			Total weight of prostates per 10 rats		
				Mean	(Range)	% change intact group	Mean, mg.	(Range)	% change intact group
Intact	none	70	7	122	(97–160)		889	(779–1159)	
Intact	2 mg. Cortisol	28	3	118	(79–132)	−3			
Adrenalectomized	none	40	4	169	(137–185)	+39	1019	(844–1143)	+14
Castrate	none	101	12	44	(33–73)	−64	155	(92–246)	−83
Castrate	0.6 mg. testosterone	50	5	138	(100–163)	+13	483	(294–646)	−46
Castrate	2.4 mg. testosterone	82	11	188	(140–290)	+54	1412	(918–2074)	+60
Castrate†	none	40	4	32	(19–45)	−74	120	(54–188)	−86
	0.6 mg. halotestin	25	4	63	(36–100)	−48	287	(164–438)	−68
	9.6 mg. halotestin	25	5	137	(95–204)	+12	762	(506–1106)	−14

* Each study represents duplicate flasks for non-treated castrates and triplicate or quadruplicate flasks on all other groups.

† These animals received their hormone by gavage; all others were given hormone by subcutaneous injection.

These animals were adrenalectomized 5 to 8 days before use and it is not certain at this time whether surgical trauma may have induced the release of some biologically active androgens from the adrenals before their removal (5). If this point can be eliminated, then it would appear that an extra-adrenal stimulation of the prostate has taken place. It was found that 2 mg. cortisol given subcutaneously to intact rats did not influence formazan titers (Table I).

The data in Table I also include the wet weights of the prostates from all animals in a particular group (10 rats per group). The expected decrease in the wet weight of prostates after castration is obvious. A slight rise in the prostate weight of adrenalectomized animals was also seen. A comparison of weight increases with testosterone and halotestin bears out the greater effect obtained with testosterone in the formazan studies.

It is of interest to note the parallel decrease in weights and reducing capability of castrates compared to intact rats. Formazan titers declined 64 to 74% while the corresponding range for wet weights was 83 to 86%. Whereas prostate weights of rats treated with 2.4 mg. testosterone exceeded intact levels by 60%, the analogous increase for TPTZ levels was 54%. The orally administered 9.6 mg. halotestin dose failed by 14% to restore weights to intact levels. On the other hand formazan titers rose 12%.

It is true that a greater difference of weight (about a factor of 10) existed between untreated and testosterone-treated castrates as compared to the corresponding changes (about a factor of 4 to 5) elicited in the titers of reducing systems. However in the case

of weights of an organ one is estimating gland enlargement possibly due to a significant extent to water and substrate mobilization. In the determination of titers of reducing systems, one is directly measuring metabolic activity of the cells as expressed primarily by dehydrogenase function. Farnsworth and Brown (6) have recently announced that early biosynthetic steps dependent upon adenosine triphosphate (ATP) may be controlled in the prostate by testosterone. The obvious source of ATP within the gland would be the dehydrogenases involved in oxidative phosphorylation.

Such biochemical approaches should more accurately pinpoint the influence of other endocrine secretions on prostatic function. Moreover a more definite bioassay procedure would be available for evaluating biological activity of compounds. An examination of these ramifications and levels of reducing systems in other tissues is underway. It has already been observed that succinic acid dehydrogenase levels parallel the increase in formazan production after testosterone treatment.

H. Rosenkrantz, V. Castle,
M. Connors and M. M. Mason

Biologics Testing Laboratory
20 Harvard Street
Worcester 9, Massachusetts

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BRAIN 5-HYDROXYTRYPTAMINE, 5-HYDROXYTRYPTOPHAN DECARBOXYLASE, AND MONOAMINE OXIDASE OF NORMAL, THYROID-FED, AND PROPYLTHIOURACIL-FED MALE AND FEMALE RATS[1]

ABSTRACT

Male and female Wistar rats (125–175 gm.) were fed for 21 days *ad libitum* diets of a meal containing 0.15% strong desiccated thyroid, 0.15% propylthiouracil, or meal alone. Neither thyroid nor propylthiouracil feeding significantly altered brain 5-hydroxytryptamine, 5-hydroxytryptophan decarboxylase, or monoamine oxidase in either sex. Female rats were found to possess significantly greater 5-hydroxytryptophane decarboxylase and monoamine oxidase activities than the males. No sex difference was noted in brain 5-hydroxytryptamine content.

Deviations from the euthyroid state produce changes in the activities of a number of enzymes (1). Such changes in the activity of monoamine oxidase (MAO), the enzyme

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responsible for the destruction of 5-hydroxytryptamine (5-HT), have been reported. Spinks and Burn (2) demonstrated that the activity of this enzyme in the livers of rats and rabbits is decreased by thyroid feeding and increased by thyroidectomy. Trendelenburg (3) also reported a decrease in the MAO activity of rabbit livers following thyroid feeding. Zile and Lardy (4), studying liver mitochondria, obtained similar results with thyroid-fed rats but found no significant alteration in MAO activity of thyroidectomized rats. Zile (5) has observed, however, that administration of thyroxine and several thyromimetic compounds causes no measureable change in brain MAO, and Dubnick *et al.* (6) that thyroidectomy does not alter brain MAO.

This report presents the results of a study of the effects of altered thyroid status on brain MAO, 5-HT, and 5-hydroxytryptophan decarboxylase (5-HTPD), the enzyme involved in decarboxylation of 5-hydroxytryptophan to 5-HT. Support is given the observations of Zile (5) and Dubnick *et al.* (6) regarding the effect of the thyroid on brain MAO activity in that brain MAO activity was not altered by either thyroid or propylthiouracil (PTU) feeding. Brain 5-HT and 5-HTPD were similarly unchanged by such treatment although data to be presented in a subsequent report show that thyroid feeding decreases and PTU feeding increases 5-HTPD activity in the heart. Although no sex difference was noted with respect to brain 5-HT levels, brain MAO and 5-HTPD activities of female rats were found to be significantly grater than those of males.

MATERIALS AND METHODS

Male and female Wistar rats (125–175 gm.) were used in this study. Wayne Lab Blox meal containing 0.15% strong desiccated thyroid (Parke-Davis) or 0.15% PTU was fed *ad libitum* to the thyroid- and PTU-fed animals respectively. Control animals received the meal alone. After 21 days the rats were killed by decapitation. Whole brains were removed, chilled, blotted dry, weighed, and homogenized in a Kontes Duall Tissue Grinder (all glass). Homogenates to be used for MAO or 5-HTPD assays were prepared with water and those for 5-HT determinations with 0.01N HCl. Histological examinations of the thyroids furnished evidence of the effectiveness of the diet.

5-HT determinations were performed by the technique of Bogdanski *et al.* (7) using an Aminco-Bowman spectrophotofluorometer; extraction of 5-HT from these homogenates was started as soon as homogenization was complete. MAO activities were assayed by the method of Sjoerdsma *et al.* (8) using 5-HT as the substrate and employing the colorimetric procedure of Udenfriend *et al.* (9); the pH of the incubation mixture was 7.5. No loss of activity was found when these homogenates were stored frozen for short periods; therefore, these homogenates were immediately frozen and stored at −15° C, the assays being made the next day. The method of Clark *et al.* (10) was used to determine 5-HTPD activity; since some loss of activity was found with standing or freezing, these assays were performed immediately. Protein content of the homogenates used for the assay of MAO or 5-HTPD were determined by the procedure of Lowry *et al.* (11).

RESULTS

Neither thyroid nor PTU feeding resulted in significant alterations in brain 5-HT, 5-HTPD, or MAO in either sex. When males were compared with females receiving corresponding diets, no significant sex difference was noted in brain 5-HT. However, mean brain MAO and 5-HTPD activities of the female groups were found to be significantly greater than those of the corresponding male groups. These results are summarized in Table 1.

DISCUSSION

Although neither thyroid nor PTU feeding alters whole brain 5-HT, 5-HTPD, or MAO, the possibility exists that such treatment might change one or more of these at specific sties within the brain.

However, while not influencing total brain 5-HT, sex appears capable of exerting an effect on brain 5-HT metabolism, and possibly norepinephrine metabolism as well, by regulating the levels of activity of 5-HTPD and MAO. In view of the greater activities of these enzymes in the female brain than in that of the male, the turnover rate of 5-HT could be expected to be more rapid in the female. Moreover, free or unbound 5-

TABLE 1. BRAIN 5-HT, 5-HTPD, AND MAO OF NORMAL, THYROID-FED, AND PTU-FED MALE AND FEMALE RATS. MEANS ± S.E.

	Normal	Thyroid-fed	PTU-fed
Brain 5-HT			
♂ (10)* μg/gm. Brain	0.43 ± 0.07	0.48 ± 0.06	0.58 ± 0.06
significance		0.60 > P > 0.50	0.20 > P > 0.10
♀ (10) μg/gm. Brain	0.40 ± 0.06	0.38 ± 0.03	0.55 ± 0.05
Significance		0.80 > P > 0.70	0.10 > P > 0.05
Significance (♂ vs. ♀)	0.80 > P > 0.70	0.20 > P > 0.10	0.80 > P > 0.70
Brain 5-HTPD			
♂ (10) Sp. Activity[a]	2.06 ± 0.06	1.96 ± 0.09	1.98 ± 0.05
Significance		0.40 > P > 0.30	0.40 > P > 0.30
♀ (10) Sp. Activity[a]	2.34 ± 0.10	2.38 ± 0.08	2.35 ± 0.11
Significance		0.80 > P > 0.70	P > 0.90
Significance (♂ vs. ♀)	0.05 > P > 0.025	0.005 > P > 0.001	0.01 > P > 0.005
Brain MAO			
♂ (10) Sp. Activity[b]	97 ± 5	93 ± 4	100 ± 5
Significance		0.60 > P > 0.50	0.70 > P > 0.60
♀ (10) Sp. Activity[b]	133 ± 2	127 ± 6	130 ± 4
Significance		0.40 > P > 0.30	0.60 > P > 0.50
Significance (♂ vs. ♀)	P < 0.001	P < 0.001	P < 0.001

* Number in parentheses represent number of rats in the group.
[a] Species Activity = μmoles 5-HT formed/gm. protein/hr.
[b] Specific Activity = μmoles 5-HT consumed/gm. protein/hr.

HT, considered to be the active form (12), would tend to be kept at a lower level in females by the greater MAO activity.

It is possible that central nervous system control of sexual development and the reproductive cycle may be mediated by 5-HT (and/or norepinephrine) and that some sort of reciprocal relationship exists between female sexual activity and brain 5-HT metabolism. The studies of Setnikar *et al.* (13) which demonstrate that administration of iproniazid, an MAO inhibitor known to increase the brain levels of these amines (14), results in changes in the sexual function of the hypophysis of the female rat suggesting a partial blockade of ICSH secretion, would support this. Additional evidence is that reserpine, which depletes brain 5-HT and norepinephrine (12), alters the estrous cycle, reduces fertility (15), and inhibits ovulation in rats (16) and can prolong the menstrual cycle (17) and prevent menstruation in monkeys (18). In an effort to determine whether such a relationship may exist, studies are now in progress to show whether brain 5-HT metabolism changes during the estrous cycle or can be altered by the administration of sex steroids.

Finally, the finding that the brain MAO activity of female rats is greater than that of males have clinical implications in relation to catamenial epilepsy and "pre-menstrual tensions." MAO inhibitors have been shown to increase the resistance of rats to electroshock seizures (19). The possibility that MAO inhibition may be useful in such conditions seems worth consideration.

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RALPH G. SKILLEN, CLINTON H. THIENES AND LORRENE STRAIN

Institute of Medical Research
Huntington Memorial Hospital
Pasadena, California

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INDIFFERENCE OF THIOURACIL-INDUCED INHIBITION OF PUBIC SYMPHYSEAL SEPARATION TO CHANGES IN ESTROGEN-PRIMING DOSAGES[1]

ABSTRACT

Present data show that increased estrogen in the priming of virgin mice fed thiouracil did improve, slightly, the pubic symphyseal response to relaxin. However, in no instance did the responses ever equal those of control mice. It is concluded from this, and earlier studies (1, 2, 3), that the inhibition of interpubic ligament elongation in thiouracil-fed mice is not simply a result of sub-optimal estrogen priming.

Previous data (1, 2, 3) suggest that thyroid hormone is necessary for the growth and maintenance of interpublic ligaments whether resulting from pregnancy or the administration of relaxin in mice. It is further indicated by these data that inhibition of symphyseal separation observed in thiouracil-fed gravid and non-gravid relaxin-treated mice is a direct result of decreased thyroid gland activity. However, it is well known that the effectiveness of estrogen is reduced in hypothyroid mice. This strongly suggests that the previously observed thiouracil-inhibition of the symphyseal response to relaxin

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TABLE 1. INFLUENCE OF VARIOUS DOSAGES OF ESTROGEN UPON INTERPUBIC LIGAMENT LENGTHS IN MICE FED THIOURACIL OR THYROID POWDER AND GIVEN A CONSTANT DOSAGE OF RELAXIN (40 μg)†

	Average interpubic ligament lengths in mm.					
	Estrogen priming dosage					
Dietary regimen	2.5 μg	5.0 μg	7.5 μg	10.0 μg	12.5 μg	15.0 μg
Fox Chow (Controls)	1.41 ±0.06*(29)**	1.78 ±0.03 (38)	2.03 ±0.09 (29)	1.66 ±0.04 (38)	1.43 ±0.20 (19)	1.18 ±0.07 (28)
Fox Chow +0.5% Thiouracil	0.32 ±0.08 (20)	0.33 ±0.07 (37)	0.45 ±0.03 (29)	0.51 ±0.05 (40)	0.80 ±0.11 (17)	0.43 ±0.09 (30)
Fox Chow +1.0% Thiouracil	0.35 ±0.17 (11)	0.33 ±0.19 (31)	0.31 ±0.08 (21)	0.32 ±0.10 (29)	0.43 ±0.15 (11)	0.48 ±0.07 (31)
Fox Chow +0.5% Thyroid	1.90 ±0.11 (19)	2.34 ±0.12 (20)	2.38 ±0.18 (11)	2.10 ±0.19 (11)	1.97 ±0.15 (10)	1.70 ±0.18 (11)
Fox Chow +1.0% Thyroid	1.93 ±0.10 (17)	2.23 ±0.16 (21)	2.42 ±0.14 (19)	1.98 ±0.17 (18)	2.07 ±0.12 (10)	2.10 ±0.11 (17)

† All mice were fed the various diets for 44 days. On the 35th day one of the above dosages of estrogen (ECP) was administered, followed by 40 μg relaxin on day 43. All mice were killed on day 44.

* Standard error of the mean.

** Numbers in parentheses indicate number of mice in group.

might be simply a result of sub-optimal estrogen priming. The present paper presents evidence contrary to such suggestion.

MATERIALS AND METHODS

Virgin mice of the Albany strain (100–130 days old) were maintained, *ad libitum*, on Purina Fox Chow, with and without supplements of thiouracil (0.5% and 1.0%) or thyroid powder (0.5% and 1.0%), and tap water for a period of 44 days. On the 35th day of that period, mice, representative of each of the dietary regimen, were given, subcutaneously, estradiol cyclopentyl-propionate (ECP) in amounts of either 2.5, 5.0, 7.5, 10.0, 12.5 or 15.0 μg. On the 43rd day all mice, regardless of the dosage of the estrogen prime, were given 40 μg relaxin[2] in 0.2 ml. of 1% benzopurpurine 4-B.[3] Twenty-four hours later (day 44) all mice were killed by cervical dislocation and interpubic ligaments were measured by the transillumination method (4).

RESULTS AND DISCUSSION

Results in Table 1 reveal that the optimum dosage of estrogen (ECP) for priming mice (*controls*) in our colony for the maximum pelvic response to a given dosage of relaxin (40 μg) is 7.5 μg. Dosages higher than this appear to decrease the response. This is in close agreement with data reported earlier by Steinetz *et al.* (5). Similarly, the best responses to relaxin were obtained in thyroid-fed animals (0.5% and 1.0%) when provided 7.5 μg estrogen. On the other hand, the maximum response to 40 μg relaxin was not observed until the dosage of estrogen reached 12.5 μg in mice fed 0.5% thiouracil and 15.0 μg for those mice fed 1.0% thiouracil. Estrogen in quantities greater than 15.0 μg were not employed since a 50% reduction in the response to relaxin occurred in mice fed 0.5% thiouracil when the prime exceeded 12.5 μg. A similar decrease in response was anticipated in mice fed 1.0% thiouracil, at high estrogen dosages, before the interpubic ligament could reach a length equal to that of *control* mice. These data substantiate those reports that the effectiveness of estrogen is decreased in hypothyroid mice. However, in no instance, regardless of the dosage of estrogen administered as a prime, was the response of thiouracil-fed mice equal to even the poorest response seen in *control* animals. It is concluded from this that the previously reported inhibition of pubic symphyseal separation in hypothyroid mice given relaxin (1) is not merely the result of an insufficient estrogen-prime.

EUGENE H. HORN AND MARTIN BARANDES[4]

Department of Anatomy
Albany Medical College of
Union University
Albany, New York

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[2] Releasin preparation W1164-A, Lot M43, each mg. equivalent to 0.12 μg relaxin Reference Standard W1164-A, Lot 8; generously provided by Warner-Lambert Research Institute, Morris Plains, N. J.

[3] Kindly supplied by Dr. Steinetz, Warner-Lambert Research Institute.

[4] Presently a third year medical student, Albany Medical College of Union University, Albany, N. Y.

THE CORPUS LUTEUM AND THE SACRAL PARASYMPATHETICS[1]

ABSTRACT

The data presented indicate that the endometrium acts as an organ, and in so doing is directly involved in the regression of the post-ovulatory corpus luteum. This activity of the endometrium is negated by section of the sacral spinal cord, which effectively denervates the motor secretory stimulation of the endometrial glandular epithelium.

INTRODUCTION

Loeb (1) demonstrated the importance of the uterus to the life of the corpus luteum and hence to the cyclic activity of the ovary. Little has since been done in the way of analysis of this unique phenomenon. It has been shown that if the uterus of the guinea pig was left *in situ*, but the blood and nerve supply sectioned on the sixth post ovulatory day, the resultant corpora would be retained more than 100 days with little or no intervening sign of estrus (2). Unfortunately the uteri from these animals were not saved for study of their histology.

Experiment

Eight young adult female rats were put into an experiment, wherein the spinal cord was sectioned between the lowest lumbar vertebra and the sacrum. The animals were killed some two months later and the ovaries and uteri were saved for study. Vaginal smears were not taken. The animals remained in reasonable good health even though the nerve supply to the urinary and anal sphincters had been sectioned.

RESULTS

At autopsy, in each of the animals it was found that both ovaries exhibited a large number of corpora lutea, probably of about four different age groups. Only a few follicles were found and these were smaller than the size ordinarily considered to be "ovulatory" (Fig. 1). The uterus was comparatively small in size but had a very characteristic appearance. The endometrium was somewhat variable, from low to a highly developed columnar type, but the glandular development was minimal. The myometrium, inner layer, was loose and spongy in nature and somewhat hyperemic (Figs. 2 & 3). One uterus contained a large growth in the myometrium. No significance is attached to this

FIG. 1. Cross section of ovary of rat 2 months after cutting of sacral spinal cord. ×12.5.

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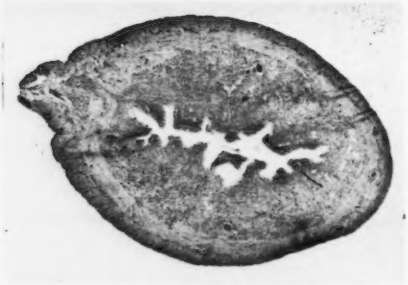

FIG. 2. Cross section of uterus of rat 2 months after cutting of sacral spinal cord. ×17.

finding other than to record its presence (Fig. 4). The growth did not appear to be malignant nor was it considered to be a deciduoma. Nerve stains did not demonstrate in any uterus any nerves near the endometrium or the myometrium. Occasional nerve fibers were found in or near the outer serous coat of the uterus.

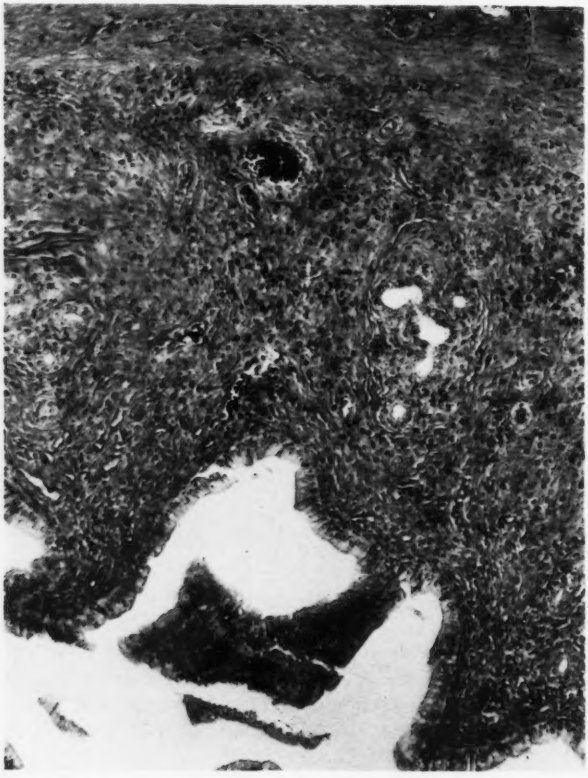

FIG. 3. Endometrium and inner myometrium of rat uterus 2 months after section of sacral spinal cord. ×105.

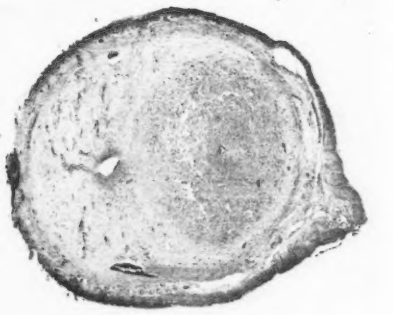

FIG. 4. Cross section of uterus containing growth 2 months after cutting of sacral spinal cord. ×17.

In the guinea pig the retention of the corpus after hysterectomy and the function of those retained corpora are both well demonstrated (1, 2, 3). In seeking an explanation for the cause of retention of corpora lutea, it seemed logical to leave the uterus intact but to alter its function in some fashion. This was first done in 1956 (2), but at that time the blood supply and the nerves to the uterus were both sectioned. Obviously it would be preferable to section the nerves and not to disturb the blood supply. To this end we cut the sacral spinal cord as described. This was done with the knowledge that the glandular epithelium of the uterus must receive its motor secretory stimulus through the parasympathetic fibers of the sacral spinal cord. The profound change in the ovary and in the uterus, following cord section, proves our contention that the changed function of the endometrium in some fashion mediates the regression of the corpus luteum. The presence of functional corpora in an ovary is most likely the source of inhibition of the development of follicles and ovulation in normal fashion. In the cases presented here, some ovulation with resultant corpora either took place or corpora were formed from anovulatory follicles.

We believe these findings offer significant information, and perhaps breakthrough information, as to why hysterectomy allows or causes the continued long life of the corpus luteum. We would point out that the finding of an abnormally large number of corpora in the ovaries of these animals is a picture not greatly different than that obtained after section of both vagal nerves in the thorax of the rat (2). The mode of action in these cases may be greatly different but the results have a similar appearance. It may be that the similarities are mediated by central nervous system nuclei or pathways

From these data we postulate that denervation (parasympathetic) of the uterus causes a basic change in the glandular functions of the uterus and the resultant change of uterine physiology exerts its influence on the life of the corpus. This may be (a) a direct effect by virtue of presence or absence of an enzyme, a metabolite or a hormone which is liberated or changed by the endometrium and humorally borne to the end organ—the corpus luteum; (b) an indirect effect, with the same type of substance acting on some portion of the intervening nervous system, endocrine system or the ovary other than the corpus luteum; (c) or, since section of vagus and sacral parasympathetics produces a similar ovarian respone, perhaps these two portions of the nervous system constitute a common mediating pathway.

The next step appears to be the preparation of a discreet extract of the endometrium, which when applied at the proper time in the life of a corpus luteum in a hysterectomized guinea pig, will cause its involution.

We further suggest that experiments having to do with denervation of the sex accessory organs of the male may be of importance relative to gonadal function.

R. Towner Hill and Morton Alpert

Division of Research Grants
National Institutes of Health
Bethesda, Md.
and
Department of Anatomy
Indiana University
Indianapolis, Indiana

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THE RELATION OF ACID PHOSPHATASE ACTIVITY OF PITUITARY GONADOTROPHS AND ACIDOPHILS TO SECRETORY ACTIVITY IN THE RAT[1]

ABSTRACT

The acid phosphatase activity of rat pituitary gonadotrophs and acidophils was studied histochemically in various phases of secretory activity, and noted to parallel hormone secretion.

Utilizing a new azo-dye method (1) for the demonstration of acid phosphatase activity, it was shown (2) that this enzyme appears to be localized in the Golgi zone of the anterior pituitary and thyroid acinar cells of the rat, and parallels hormone secretion histochemically in pituitary thyrotrophs and thyroid. This was confirmed chemically for the thyroid. To determine whether this was a more general phenomenon, the acid phosphatase activity of pituitary gonadotrophs and acidophils in various phases of secretory activity are reported in the following.

MATERIAL AND METHODS

Carworth Farms CFE (Sprague-Dawley descendant), male, albino rats of approximately 120 gm. were kept 5 in a cage and had free access to water and food (Rockland Rat Pellets). Groups of ten animals each were either injected subcutaneously daily with 10 mg. (5 U.S.P. units) of growth hormone (Raben Type), or received by the same route 1 mg. of testosterone (malestrone) twice weekly, or were castrated. The animals were killed by exsanguination under ether anesthesia simultaneously with controls at 3, 4 and 5 weeks respectively.

The pituitary glands of all rats were fixed for 24 hours in cold formol calcium, pH 7.0. Frozen sections were stained by an azo-dye method (1) for acid phosphatase, and parallel sections with hematoxylin and eosin and PAS. The criteria previously stated (2) were utilized in differentiating the cells of the adenohypophysis.

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RESULTS

After testosterone treatment the gonadotrophs revealed decreased cytoplasmic PAS staining, and a smaller negative image of the Golgi apparatus. The latter contained considerably less acid phosphatase activity (Fig. 2) than the corresponding oval paranuclear area in the control rats (Fig. 1). The dye deposit was more finely granular, and the surrounding cytoplasm contained almost no activity.

Following castration the gonadotrophs increased in number and size revealing more cytoplasmic PAS staining, and occasional vacuolization. The negative image of the Golgi zone was larger and contained markedly increased and coarser dye deposits (Fig. 3) which were also noted in the surrounding cytoplasm.

After growth hormone treatment, the only change noted was a diminution of acid phosphatase activity (Fig. 4) and finer granularity of the dye deposit in the semilunar paranuclear areas of the acidophils.

DISCUSSION

The acid phosphatase activity of the rat pituitary gonadotrophs and acidophils was altered characteristically by treatments which have been shown to change the secretory activity (hormone synthesis and/or release) of these cells (3–7). As noted for the thyrotrophs and thyroid acinar cells (2), acid phosphatase activity of gonadotrophs was increased in states of increased secretory activity, and conversely that of gonadotrophs and acidophils was found to be decreased with diminished secretion. These observations seem to indicate a relationship between acid phosphatase activity and hormone synthesis and/or release, and possibly represent a general cytological phenomenon.

All photomicrographs are of uncounterstained acid phosphatase preparations of rat pituitary gland at a magnification of 480×.

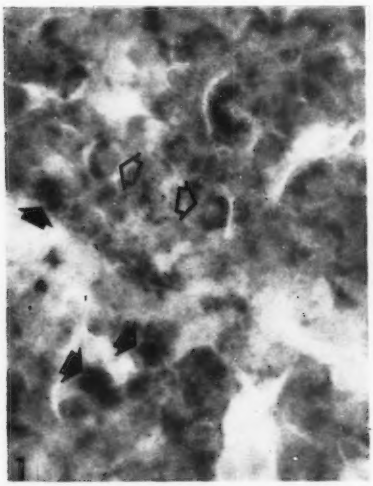

FIG. 1. Control showing prominent oval areas of staining in basophils (closed arrows) and semilunar areas in acidophils. There is a fine granular azo dye deposit in the surrounding cytoplasm. Prominent negative image of nucleus is noted in two acidophils (open arrows).

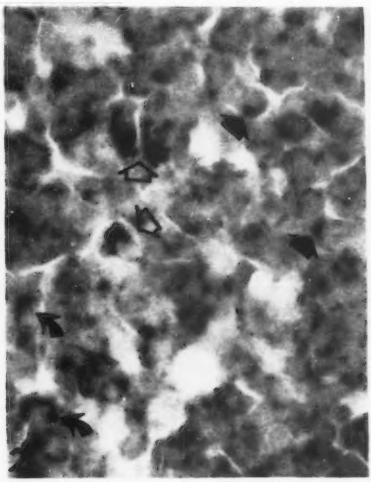

FIG. 2. Following testosterone treatment diminished size and density of azo dye deposits in gonadotrophs is noted (closed arrows). Surrounding cytoplasmic granularity is virtually absent. Thyrotrophs (open arrows) and acidophils (curled arrows) are unaltered.

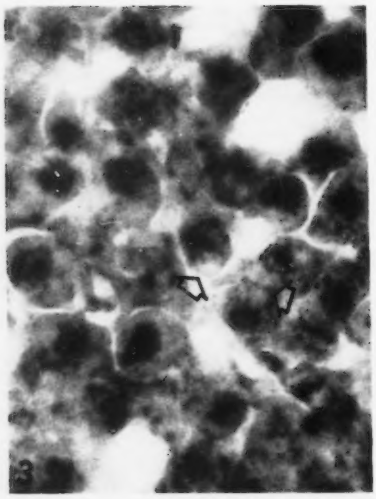

FIG. 3. Following castration note increased size and density, and coarser granularity of oval dye deposits in enlarged gonadotrophs. Prominent negative image of nucleus is noted at arrows.

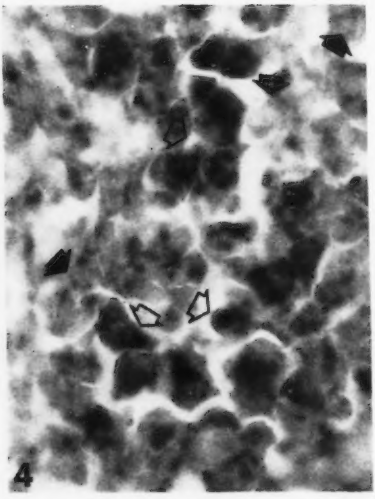

FIG. 4. Following treatment with growth hormone note diminished staining of acidophils (closed arrows). Basophils (open arrows) are unaltered.

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HAROLD J. SOBEL[2,3]

Department of Pathology
The Mount Sinai Hospital
New York, N. Y.

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[2] Trainee in Experimental Pathology, U.S.P.H.S., N.I.H.

[3] The author's present address is Department of Pathology, The Albert Einstein College of Medicine, New York 61, N. Y.

NEURONAL DEGENERATION, ELECTROENCEPHALOGRAPHIC DISTURBANCES AND POLYSEROSITIS IN RATS PRODUCED BY IMINODIPROPIONITRILE AND PREVENTED BY l-THYROXINE

ABSTRACT

Paresis and/or paralysis, EEG disturbances and polyserositis were observed in rats after prolonged administration of β-β'-iminodipropionitrile (IDPN). Rats treated with thyroxine simultaneously to the administration of IDPN did not show paralysis or polyserositis and the electroencephalographic pattern of this group was completely normal whereas the intoxicated animals showed electroencephalographic abnormalities. Histopathological examination of the spinal cord demonstrated that IDPN induces basophilic degeneration of the anterior motor horn cells. Thyroxine also prevents this neuronal degeneration. The practical significance of this protection is pointed out.

Multiple forms of lathyrism can be induced in rats by the administration of aminonitriles of varying chemical composition. BAPN,[1] AAN[1] and MAAN[1] produce bone lesions (osteolathyrism) (1) (2) (3), whereas IDPN[1] provokes neurological manifestations (neurolathyrism) which are characterized either by the ECC syndrome (Excitation, Choreiform and Circling movements) in acute poisoning (1) or by paralysis and polyserositis in chronic intoxication (4). It has been observed that thyroxine protects completely against the appearance of the ECC syndrome (5) (6). The present report describes the effect of thyroxine on the clinical picture, EEG patterns and pathological changes of the spinal cord in rats after prolonged administration of IDPN.

MATERIAL AND METHODS

Twenty-five rats of the Wistar strain, 60 days old, weighing 85 to 110 gm and almost equally distributed as to sex, were distributed into three groups. Group 1 with five rats served as intact controls. Ten rats of Group 2 were given subcutaneous daily injections of 15 mg. of IDPN in 0.2 cc. of water during 13 weeks. To the ten rats of Group 3 sodium l-thyroxinate at a daily dose of 200 μg of thyroxine in a 0.2 cc. of a slightly alkaline aqueous solution was injected subcutaneously throughout all the experiment starting 8 days prior to the administration of IDPN. This nitrile was also given to Group 3 in the same dose and by the same route as Group 2 for 13 weeks. Animals were housed in groups of 4 rats each, weighed weekly and carefully observed during all the experiment. The rats of all three groups were maintained exclusively on a purina chow standard diet and water *ad libitum*.

We considered the "swimming test" as the most appropriate one to determine the initiation of the ECC syndrome. The swimming test was repeated every second day from the beginning of the experiment. When placed in water, normal rats swim with coordinated movements. The affected animals do not swim but perform rotatory movements and are unable to keep from drowning. This behavior is called "barrel rolling." Paresis and paralysis were detected by careful inspection of the gait pattern. The existence of ascitis was discovered by inspection and evaluated by postmortem examination.

Three weeks after the onset of IDPN administration EEG records were taken. Rats were anesthetized with Nembutal. Contact unpolarizable electrodes consisting of a silver chloride thread were sutured into the skin of the skull at the points marked in Figure 1. A ten channel "Petit" electroencephalograph was used, recording simultaneously three rats of Groups 1, 2 and 3 thereby utilizing only two channels for each animal. Rates of 30 mm./sec. and amplification of 50 μV = 0.5 cm. were employed.

Rivalta reaction and protein content were determined in the ascitic fluid. Surviving animals were sacrificed by ether inhalation at the end of the experiment. The spinal cord and the kidneys were immediately dissected and fixed in 10% formalin for 10 days. After fixation, part of the tissues were embedded in paraffin and others were cut by freezing. Sections were stained with hematoxylin and eosin.

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[1] In the present paper the following abbreviations are used: BAPN: betaaminopropionitrile; AAN: aminoacetonitrile; MAAN: methylenaminoacetonitrile; IDPN: β-β' iminodipropionitrile.

TABLE 1. EXPERIMENTAL NEUROLATHYRISM PRODUCED IN RATS AFTER CHRONIC ADMINISTRATION OF β-β′ IMINODIPROPIONITRILE (IDPN)

No.	Group	No. of rats	Paresis and/or paralysis	Polysero-sitis	Positive swimming test %	EEG	Spinal cord pathological findings
1	Normal	5	0	0	0	Normal	Negative
2	IDPN[1]	10	3+	3+	100	Abnormal	Positive
3	IDPN[1] + Thyroxine[2]	10	0	0	30	Normal[3]	Negative

[1] 15 mg. daily.
[2] 200 μg daily.
[3] Excepting the three rats with positive swimming test.

RESULTS

The results of these experiments are summarized in Table 1. All the animals of Group 2 showed a positive "swimming test" by the 19th day after the administration of IDPN, whereas all the animals of Groups 1 and 3 were completely normal at that time. However three weeks later, 3 animals (30%) of Group 3 showed a positive "swimming test" which remained evident until the end of the experiment. Paresis was observed in all rats of Group 2 at the 8th week, increasing in severity during the following weeks and by

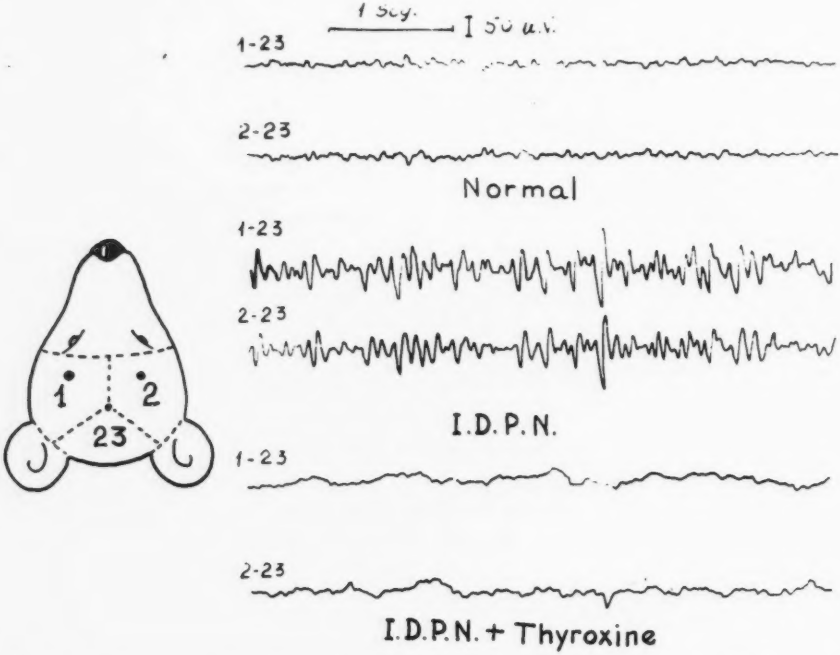

FIG. 1. EEG patterns of rats; normals, treated with IDPN and protected with thyroxine.

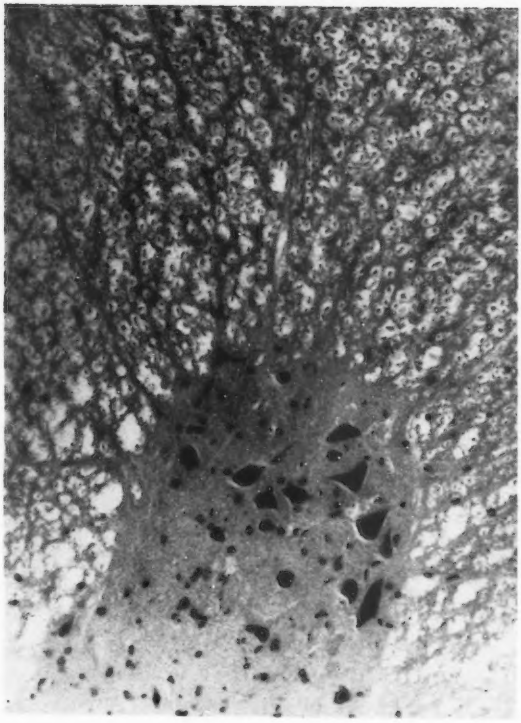

FIG. 2. Normal aspect of anterior motor horn of the spinal cord of rats of group 3 treated simultaneously with IDPN and thyroxine (H&E ×100).

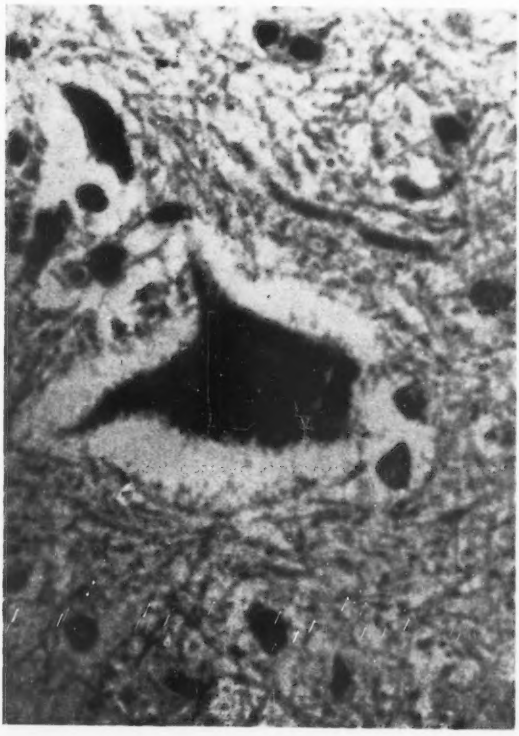

FIG. 3. Rat of group 3. Normal neuron. Higher magnification. (H&E ×400).

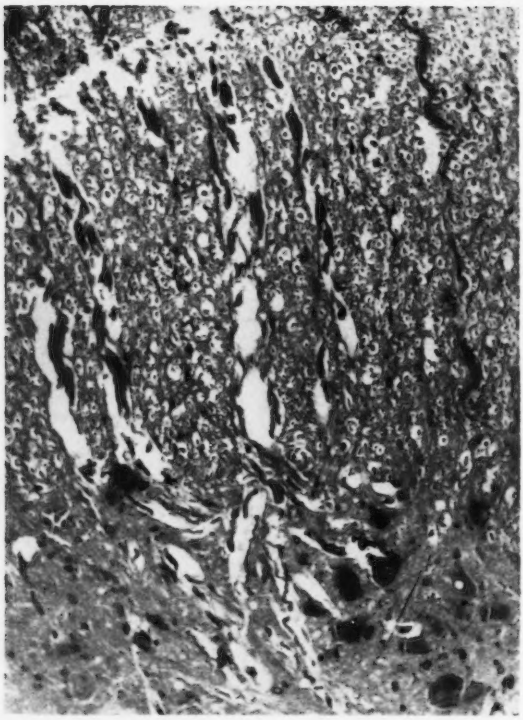

FIG. 4. Degeneration and fragmentation of intraspinal portion of the motor axon and of nerve cells in a rat of group 2 with chronic IDPN poisoning. (H&E ×100).

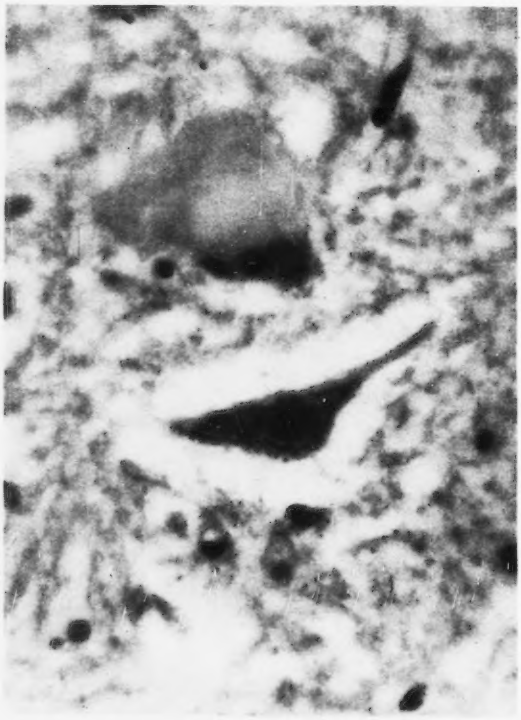

FIG. 5. Rat of group 2. Neuron transformed into an amorphous basophilic mass. (H&E ×400).

the 13th week two animals showed complete paralysis of the hind limbs. None of the animals of Groups 1 and 3 presented neuromotor disturbances. On the other hand all the rats of Group 2 develop a generalized polyserositis affecting both pleural and peritoneal cavities. Rivalta reaction was strongly positive in the peritoneal fluid in all the animals investigated. The average protein content of the exudate was 26 gm.% (5 rats). Mortality was very high in this group, amounting to 60% of the animals. Hyperemia and petechial hemorrhages of mesenteric vessels and an increase in size of the mesenteric lymph nodes were also seen. Animals of Groups 1 and 3 did not show these alterations and there was no mortality. The bones were normal in all groups.

The EEG patterns of control rats (Group 1) and of those protected with thyroxine (Group 3) were very similar. Rats of Group 2 however, showed a more rapid basal record than normals. This activity was followed by bilateral synchronous discharges of sharp and theta waves of 4 cycles/second. (Fig. 1).

The result of the histopathological study demonstrated that rats of Group 3 did not show histological lesions of the spinal cord. (Figs. 2 and 3). In Group 2 the most marked microscopic lesions were seen in the anterior horn of the spinal cord (Fig. 4). The intraspinal portion of the axons of the anterior motor horn cells showed severe basophilic degeneration which in some instances progresses to complete disintegration. Numerous neurons in the same area (ventrolateral nucleus) exhibited disintegration of the nucleus, disappearance of the nucleolus, tigrolysis and cytolysis. Some neurons are found transformed into an amorphous basophilic mass. (Fig. 5). The kidneys were normal both macro- and microscopically in all groups.

DISCUSSION

After the prolonged administration of IDPN (15 mg. daily for 13 weeks) rats develop paresis and/or paralysis and polyserositis and subsequently a high mortality rate. These symptoms are preceeded by the appearance of the acute ECC syndrome and alterations of the EEG pattern, which suggest a functional disturbance of hypothalamic centers although we have at present no evidence of morphological lesions.

Polyserositis does not appear to be the consequence of renal damage since the histological examination of the kidneys was normal. The positive Rivalta reaction and the high protein content of the ascitic fluid pointed out that the latter is an exudate, probably a result of changes in capillary permeability and/or lymphatic circulation.

The production of neurological lesions in the anterior horn cells of the spinal cord by the administration of IDPN shows that this aminonitrile has a direct neurotoxic action. This effect is probably more widespread in the nervous system, as the EEG disturbances seem to support. These histological changes of the nerve cells were also observed by Hartmann, Lalich and Akert (7). They attributed the neurotoxic effects to the presence of two nitrile groups in the molecule of IDPN.

Thyroxine prevents the bone lesions produced by AAN and MAAN (1) (3) (8). It also protects against the neurotoxic alterations induced by IDPN. It is interesting to consider the difference of tissue types, namely connective tissue and nerve cells, that are capable of being affected by aminonitriles and protected by thyroxine. The mechanism of thyroxine in protecting aminonitrile intoxicated rats has been studied (9) but it is yet not clearly elucidated.

It may be interesting to consider in relation to these experimental results the recent observation of Rawson *et al.* (10) who related the improvement of certain mental conditions to the administration of thyroxine and also the relationship between the thyroid gland and the nervous system as pointed out by Eayrs (11) in a recent review. The similarity between the chronic IDPN poisoning and human lathyrism is also striking.

Acknowledgments

The authors are indebted to Dr. M. Morales for his advice on the histopathological interpretation and to Dr. C. Oliveros for his evaluation of the EEG studies.

FRANCISCO VIVANCO, JOSÉ A. SANCHEZ-MARTIN AND CARLOS JIMENEZ DIAZ

Dept. of Nutrition and Hormones
Instituto de Investigaciones
Clinicas y Médicas
Madrid University, Spain

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CHEMICAL STERILIZATION OF MALE RHESUS MONKEYS

ABSTRACT

Male Rhesus monkeys were sterilized by a single intratesticular injection of cadmium chloride. The seminiferous epithelium was totally destroyed but the interstitial elements regenerated after an initial phase of atrophy.

A single subcutaneous injection of $CdCl_2$ has been reported to cause acute degenerative changes in the testis of rats (1, 2). The seminiferous epithelium is permanently destroyed but the interstitial elements regenerate after an initial phase of disorganization. The animals became sterile as early as 24 hours after administration of the salt (3). It has been observed that these effects could be evoked by a considerably reduced dose if $CdCl_2$ was injected *directly* into the testis (4). These findings led to an exploration of the possibility of sterilizing male Rhesus monkeys by intratesticular administration of $CdCl_2$. A preliminary account of this study is presented in this communication.

TABLE 1. THE TESTIS WEIGHT OF RHESUS MONKEYS AFTER CADMIUM CHLORIDE ADMINISTRATION

Monkey No. and body weight (kg.)	Dose and site of $CdCl_2$ injection	Days after $CdCl_2$ injection	Testis weight (gm.)	
			Right	Left
K1 (10.5)*	Distilled water—*both testes*	40	20.5	20.0
K5 (9.6)	10 mg./kg. —*left testes*	3	14.0	96.0
K4 (12.5)	10 mg./kg. —*both testes*	5	65.0	60.0
K2 (7.0)	5 mg./kg. —*both testes*	40	18.0	16.0
K3 (8.7)	2.5 mg./kg. —*both testes*	75	4.8	4.2

* Body weight.

MATERIALS AND METHODS

Adult Rhesus monkeys were procured from a local dealer and kept under standardized laboratory conditions for several months before commencement of this investigation. A single injection of $CdCl_2$ (1% solution) was given *directly into the testis* under strict asepsis. The doses and sites (left or both testes) of administration are given in Table 1. In experiments where both the testes were involved the half of the total amount of $CdCl_2$ calculated on body weight basis was injected into each testis. The control animal (Kl, Table 1) received sterile distilled water alone (5 ml. per testis) in a similar manner. The testes were removed surgically and fixed in Bouin's fluid after recording their weight. The serial paraffin sections were stained with Ehrlich's hematoxylin and eosinol.

RESULTS

The testis of the control monkey (K1) presented normal macroscopic and microscopic features (Fig. 1). The administration of $CdCl_2$ (10 mg., 3 days, K5, Table 1) caused a marked increase in size of the left testis; the right one was normal macroscopically and histologically. There was severe disorganization of the tubules in the left testis. Extensive necrosis and exfoliation of the seminiferous elements were consistently

FIG. 1. The testis of the control monkey (K1) ×120. Note normal condition.

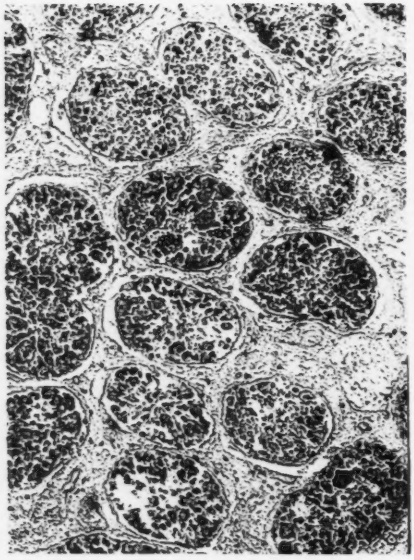

FIG. 2. Left testis of the monkey (K5) injected with $CdCl_2$ (10 mg./kg.)×120. Note tubular degeneration and disorganization of the interstitium. The right testis of this animal was normal.

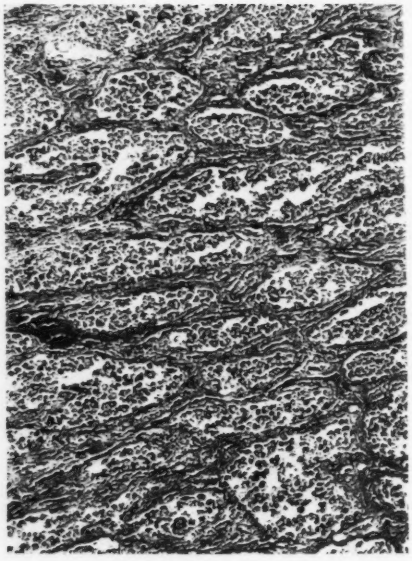

FIG. 3. The testis of the monkey (K2) injected with 5 mg./kg. $CdCl_2$×120. Note total necrosis and marked shrinkage of the tubules. The seminiferous epithelium is a debris of dead cells. Peritubular fibrosis is evident.

observed (Fig. 2). The tunica propria was degenerated. The interstitium was edematous and hemorrhagic; the cellular elements were atrophic. Focal inflammatory changes were seen throughout the interstitium. The same dose of $CdCl_2$ (10 mg., 5 days, K4, Table 1) injected into each testis evoked more or less similar change. At the 5 mg. dose (K2, 40 days, Table 1) there was some reduction in weight. The histological examination showed total necrosis and marked shrinkage of the tubules (Fig. 3). There was peritubular fibrosis. The tunica albuginea was thickened and fibroblast-like cells proliferated underneath this layer, migrated into the peripheral interstitial areas. However, few Leydig cells were seen. The more central portions of the interstitium contained only occasional fibroblast-like cells. The proliferation of new blood vessels in the intestitium was also a noteworthy feature. In the monkey K3 (2.5 mg., 75 days) the testes were markedly atrophied and hard in texture. The histological features were more or less similar to those of the monkey K2 (5 mg.). However, the peritubular fibrosis was considerably reduced and the interstitium presented normal features with many healthy Leydig cells and numerous fibroblast-like elements.

DISCUSSION

The results of the present preliminary study suggest that the male Rhesus monkeys could be sterilized by a single intratesticular injection of $CdCl_2$. Further, the nature and sequence of changes evoked in the testis are comparable to those observed in rats (1, 2). Thus in the acute stage there is disorganization of the seminiferous epithelium

and degeneration of the tunica propria. The interstitium becomes edematous and hemorrhagic; focal inflammation and mass atrophy of the cellular elements are also of common occurrence. This stage is followed by one of *total* necrosis and marked shrinkage of the tubules; the seminiferous epithelium degenerates completely. The testis becomes markedly atrophied. However, the changes in the interstitium are transitory and its histoarchitecture is eventually re-organized. New Leydig cells, presumably differentiated from the fibroblast-like elements are also consistently encountered. The net result of $CdCl_2$ administration to the Rhesus monkey is, therefore, a complete destruction of the seminiferous epithelium; but eventual regeneration of the interstitium after an initial phase of atrophy. Detailed studies are in progress.

AMIYA B. KAR

Central Drug Research Institute
Lucknow, India

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THE ENDOCRINE SOCIETY

1962 Annual Meeting

The Forty-Fourth Annual Meeting of The Endocrine Society will be held in the Palmer House, Chicago, Illinois, Thursday, Friday and Saturday, June 21-22-23, 1962.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 22 at 7:30 P.M. preceded by cocktails at 6:30 P.M.

The Chairman of Local Arrangements is Dr. Allan T. Kenyon.

All members are urged to make their hotel reservations immediately. The Palmer House will hold 300 bedrooms for members until May 1, 1962, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel advising them of time and date of arrival and departure. If you plan on remaining for the A.M.A. Meeting, please advise the hotel. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1962.

Everyone attending the meetings is required to register. By action of the Council, non-members will be charged a registration fee of $15.00 for any part of the scientific sessions. Members will be charged a registration fee of $5.00.

Graduate students, residents and interns and members of the medical departments of the armed forces will be admitted upon presentation of proper credentials CERTIFIED BY DEPARTMENT HEAD OR SUPERINTENDENT OF HOSPITAL and payment of $5.00 registration fee.

Final program, membership card and advance registration forms will be sent on May 15, 1962 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Leslie L. Bennett, Dept. of Physiology, University of California Medical Center, San Francisco 22, California, not later than February 15, 1962. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas, graphs or extensive tables cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5 filing cards, one for each author, giving his name and initials in the upper right hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors and mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.
6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Bennett.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1962 AWARDS

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch

Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958, Dr. Wilbur W. Swingle in 1959, Dr. Emil Witschi in 1960 and Dr. Lawson Wilkins in 1961. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, 1959 Dr. Gordon L. Farrell, 1960 Dr. Don H. Nelson and 1961 Dr. Ernst Knobil. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which

will not exceed $7,500 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Awards

The Council of The Endocrine Society has approved a change in status of the Upjohn Scholar to that of The Upjohn Awards.

There will be two awards in the amount of $1,250 each, one for the best article in format and content printed in ENDOCRINOLOGY and one in THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM during the preceding year.

Awardees will be chosen by the Awards Committee at its regular meeting in November of each year.

NOMINATIONS

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15 each year.

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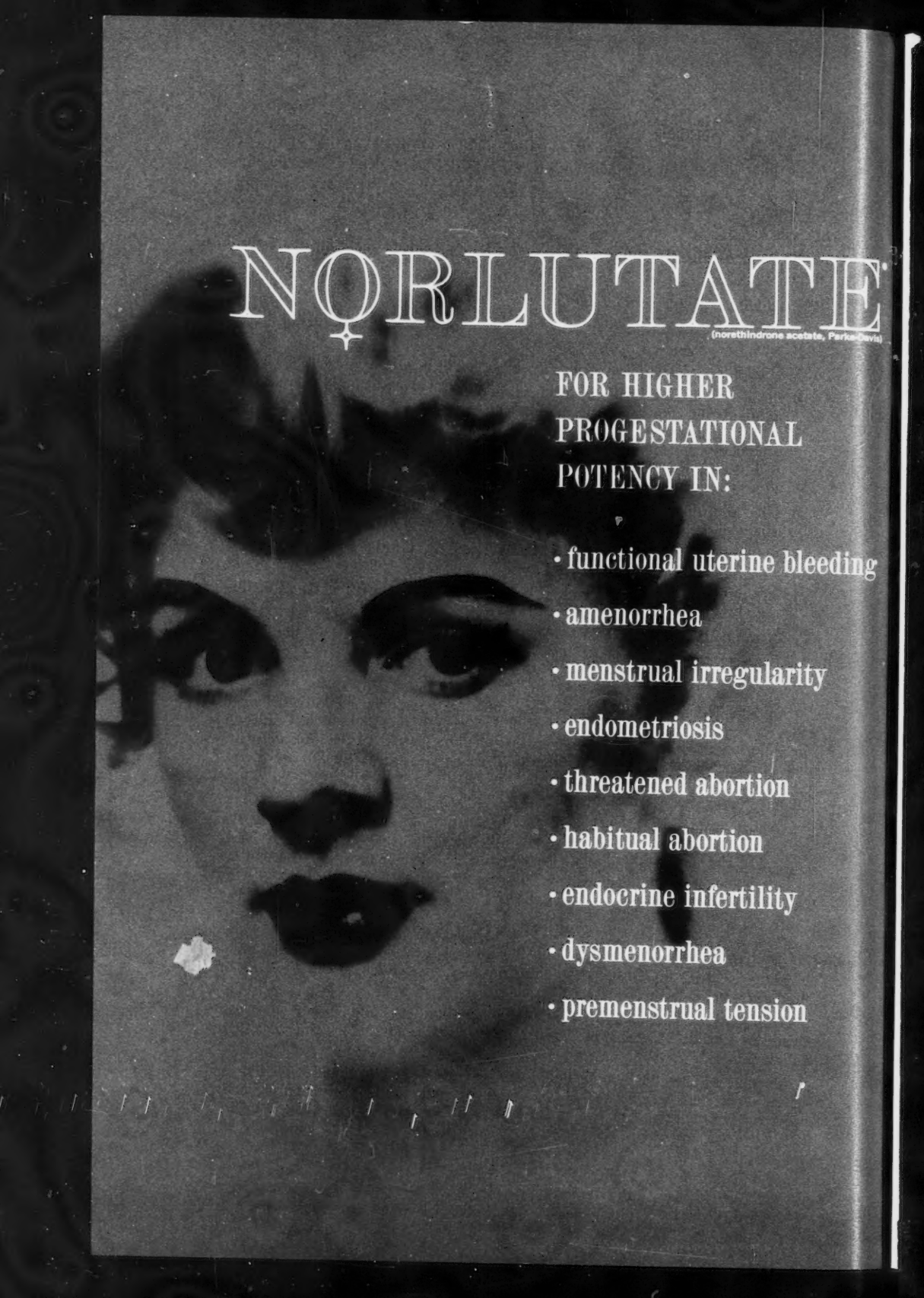
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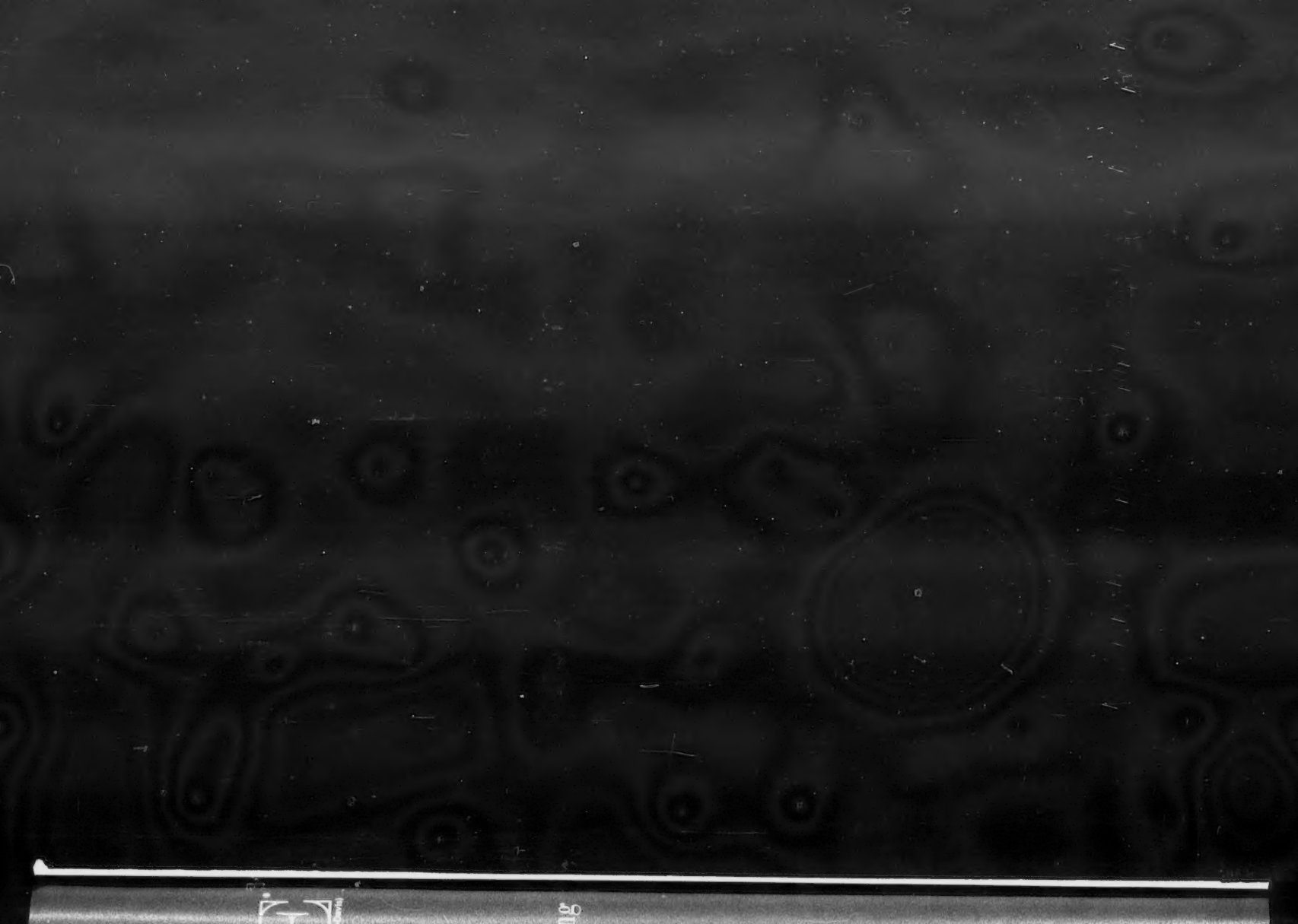

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